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Bacterial Control of Aquatic Algae

by
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Medical College of Ohio at Toledo
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BACTERIAL CONTROL OF AQUATIC ALGAE

by

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2. Mr. Thomas Stetak, B. S.; Technician.
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I. INTRODUCTION:

The concept of microbiological control of pests and pollution has been recognized for many years. A principal advantage of the natural system is that introduction of non-specific toxic chemicals into the environment is avoided. Basic ecological research and succeeding field experiments have since proven the value of microorganisms in control of unwanted populations. The use of a virus to control rabbit (Fenner, 1959) and insect (Rivers, 1964; Shea, 1971) populations has been demonstrated; and in recent years viral control of algal populations (Padan, Shilo and Kisler, 1967; Safferman and Morris, 1964, 1967; Granhall, 1972) has been partially successful.

This kind of approach, of artifically introducing a natural prey for an undesired host, provided the stimulus for investigating bacterial parasitism of algal populations.

Bacterial control of algal populations in aquatic habitats is still largely undeveloped, but initial investigations have indicated that some bacterial antibiotics can effectively reduce populations of these natural pollutants (Sladeckova and Sladeck, 1968). Recent investigations of a unique bacterium Bdellovibrio bacteriovorus, have demonstrated the effectiveness of this small microorganism in destroying populations of host bacteria (Shilo, 1969; Starr and Baigent, 1966; Burnham, Hashimoto and Conti, 1968; Starr and Seidler, 1971). The occurrence of this organism in the natural environment is widespread and well documented (Shilo, 1969; Stolp and Starr, 1963) but no one has examined the interaction that this organisms might have with the equally common procaryotic blue-green algae.

Ever since Stolp and Petzold (1962) first discovered the existence of a parasitic bacterium, investigators have been examining microbial parasitism from two approaches: one group has been involved in isolation and characterization of B. bacteriovorus from soils and sewage in an attempt to determine the economic importance of this organism in controlling the populations of susceptible Gram negative bacterial hosts (Guelin, Lepine and Lamblin, 1967); the other group has been concerned with the basic biochemical, physiological and structural characteristics of this parasitic organism and its hosts so that there might be a better knowledge of the interaction of this organism with other bacteria -- that host parasite relationships in general might be better understood.

The name Bdellovibrio bacteriovorus, first proposed by Stolp and Starr (Burnham and Robinson, 1972; Stolp and Starr, 1963), adequately expresses the principal characteristics of the organisms: "Bdello" is derived from the Greek word meaning "Leech"; "vibrio" denotes its shape; and "bacteriovorus" indicates that it literally eats bacteria. Initially it was reported by Stolp and Starr (1963) that B. bacteriovorus was an ectoparasite -- that apparently it did not enter into the host but obtained its nutrient from a position on the host cell wall. Scherff, DeVay and Carroll (1966) were the first to show that B. bacteriovorus attacks on gram negative bacteria resulted in the parasite actually penetrating into the host bacterium rather than remaining on the outside. These results were supported by Starr and Baigent (1966) and by Lepine, Guelin, Sisman and Lamblin (1967) in studying a B. bacteriovorus attacking Salmonella typhi obtained from a polluted river.

Burnham, Hashimoto and Conti (1968), investigating the penetration mechanism in detail, showed that both physical and enzymatic actions combine to cause the localized breakdown of the host cell wall.

The evidence to date, then, supports the idea that B. bacteriovorus is an endoparasite that eventually penetrates the host cell and multiplies within its confines. The cycle is completed on release of the new progeny from the host. Further refinement of the life cycle has been reported (Burnham, Hashimoto and Conti, 1970).

Until recently, B. bacteriovorus was unique in that it was the only bacterium known to parasitize other bacteria; however, Guelin et al (1968) have isolated a related organism that is able to attack and penetrate Gram positive bacterial hosts. This is extremely significant because it means that there may be other species of these very small bacteria that are capable to parasitism against other microorganisms.

II. Rationale for the Concept of Bacterial Parasitism of Algae

Ultrastructural investigations of the various species of blue-green algae (Cyanophyta) have indicated that this group is procaryotic in structural organization. This means that they are really more closely related to the bacteria than they are to the other algal forms (Chlorophyta, Chrysophta, etc.). In terms of the proposed investigation, the following facts are significant if bacterial parasitism of the algae can be considered feasible with the understanding we have at this time of the nature of bdellovibrio parasitism.

(a) the internal organization of the blue-green algae is similar to that of the bacteria, particularly those species of bacteria that

bdellovibrios parasitize (Echlin and Morris, 1965; Lang, 1968).

(b) B. bacteriovorus has been shown to parasitize photosynthetic bacteria (Rhodospirillum rubrum (Burger, Drews and Ladwig, 1968) and Chromatium strain D (unpublished information) so that the presence of photosynthetic thylakoids within the algal cells should not inhibit parasitism.

(c) Although the cell wall of the blue-green algae has been shown to be structurally distinct from that of the typical Gram negative bacteria (Lang, 1968), it has been reported that biochemically the cell walls of the two types of microorganisms are very similar (Echlin and Morris, 1965). This is particularly important in view of the observations of Varon and Shilo (Varon and Shilo, 1969) concerning the possible receptor sites for bdellovibrio attachment. Also significant as far as the receptor site are the similarities between bdellovibrio attachment and the attachment of phage to host cells (Stolp and Starr, 1963). In addition, B. bacteriovorus hosts are susceptible to bacteriophage attack. Furthermore, blue-green algal phage have been isolated which will attach to and eventually lyse species of blue-green algae (Padan, Shilo and Kisler, 1967; Safferman and Morris, 1964, 1968). All of this is evidence for a similar surface biochemistry between the bacteria and the blue-green algae.

(d) Bacterial and Algal Lysis: Antagonistic relationships between bacteria were reviewed by Stolp and Starr (1965), particularly in regard to the production of antimicrobial substances like enzymes, antibiotics and direct microbial attack upon another cell. The classic example of this latter category is the Bdellovibrio bacteriovorus described earlier. The mechanisms and enzyme interactions that explain how this parasitism

is successfully completed are still not totally understood (Starr and Seidler, 1971), but recent isolation of muramidases and proteases from bdellovibrio populations have explained some of this (Fackrell, Cambell, Huang and Robinson, 1972). These authors point out that by itself the bdellovibrio peptidase does not lyse living cells; only heat killed cells are susceptible to enzyme degradation. They further demonstrate that the site of activity was the mucopeptide layer of both the host, Spirillum serpens, and the parasite itself (Fackrell, Cambell, Huang and Robinson, 1972). Recently this enzyme has been purified and separated from protease activity completely (Dr. J. Robinson, personal communication).

Shilo and Bruff (1965) demonstrated that another species of Bdellovibrio bacteriovorus strain A3.12, was capable of degrading a variety of host bacterial species but only if they had been killed. They postulated that exoenzyme production by the bdellovibrios was the second stage of a two stage penetration process, the first being some sort of mechanical damage due to the bdellovibrio's violent attack (Burnham, Hashimoto and Conti, 1968).

Bacteriolysis is characteristic of a few other groups of bacteria, notably the myxobacteria, the cytophaga, and the actinomycetes. In studying the Myxobacter strain AL-1, Ensign and Wolfe (1965, 1966), described an enzyme possessing both proteolytic and cell-wall lytic activity. These two functions were inseparable upon purification, making the enzyme considerably different than that isolated and purified from bdellovibrios (Fackrell, Cambell, Huang, and Robinson, 1972; Dr. J. Robinson, personal communication). Review of actinomycete lysis of other microbes indicates that the responsible enzymes are peptidases

the substance might play a role in algal control in natural ecosystems (Granhall and Berg, 1972).

Since it has been shown that both Gram positive and Gram negative bacterial cell walls are penetrated by B. bacteriovorus or a related bacterium (Shilo, 1969; Starr and Seidler, 1971), it is not unreasonable to assume that the slightly different layering of the outer cell wall of most blue-green algal species will also be penetrable by a parasitic species of bacteria.

This project actually involves a search for a specific disease -- a disease of algae. The present etiologic agent, the bdellovibrio, therefore, can rapidly spread itself through an aquatic environment. This means that B. bacteriovorus may be capable of eliminating large populations of these algae. This concept of algal control by natural means has been found to be significant in both the laboratory environment and in the natural environment of the algal populations (Padan, Shilo and Kisler, 1967; Safferman and Morris, 1964). The primary problem of viral control of blue-green algae has been that the phage is too specific to species. From my experience with the B. bacteriovorus strains ATCC #15143 and UKi2, there is much greater cross-reactivity than phage possess (Burnham, Hashimoto and Conti, 1968; Shilo, 1969; Starr and Seidler, 1971). This fact will be particularly important in consideration of the type of blue-green algal species that are found in the Western Lake Erie area and in the water supplies of the state of Ohio (Palmer, 1962; Smith, 1950). These species will be the choices for experimental hosts.

II. RESULTS

A. Description of bacterial isolates lysing blue-green algae.

Because very few of the naturally algal associated bacteria present in a freshly isolated algal culture grew when struck out on a nutrient agar plate a technique of using autoclaved algae as the bacterial nutrient source was developed. First a layer of plain agar in distilled water was layered and hardened on the petri plate followed by a layer of agar containing the algae concentrated from liquid culture by centrifugation and autoclaved. This upper layer contained no other nutrient source other than the algae and necessitated the growing bacteria's obtaining all nutrients from the autoclaved algae, therefore, selecting for those bacteria which would be the best algal parasites. This method was successful in the isolation of a bacterium which could lyse blue-green algae (fig. 1). The A_{12} bacteria seen in the figure secretes a lytic enzyme which not only breaks down the cell envelope of the algal cell but several protease substrates as well. The organism appeared to be a Pseudomonas species. When Chlorococcum and this bacteria were equally mixed in liquid culture the optical density of the Chlorococcum cells at 660 nm was halved in four days. The lytic action of the A_{12} exoenzyme caused the loss of chlorophyll absorption peaks upon analysis with a scanning spectrophotometer.

One example of the frustration of working with the slow growing blue-green algae is the attempts to achieve bacterial-free cultures of our species of Oscillatoria. When my laboratory recently obtained a Zeiss Stereomicroscope with a Leitz micromanipulator I was able to isolate single cells of Oscillatoria which when placed on nutrient media did not develop colonies from contaminating bacteria. These

cells were then placed on algae agar, the normal and successful growth medium for this strain. The cells when observed by phase contrast optics showed a gradual deterioration finally resulting in their death. Apparently some factor (possibly Vitamin B_{12}) (Provasoli, 1958) is being supplied by the "contaminating" bacteria present in the normal culture without which the bacterial-free algae is not going to develop.

Actually many other methods were utilized in trying to obtain bacterial-free cultures of this strain of Oscillatoria. In addition to my own efforts, my graduate student, Miss Marge Sielicki, also applied effort in this cause as part of a "Problems Course" at Toledo University. Experiments were developed and carried out using a variety of antibiotics, filtration, differential centrifugation, and bacterial inhibitors (such as Potassium tellurite) only to have the blue-green algae in almost every case turn up more sensitive to the agent employed than the bacteria. Much of the algal literature for obtaining bacterial-free cultures of algae, relates that single cell isolation by micro-manipulation is often the most successful method (Jackson, 1964). Because of this difficulty, most of the experiments involving the Oscillatoria species were done using what should be called "minimal contamination" obtained by growing the Oscillatoria in liquid and then differentially centrifuging the cells before use, thereby eliminating most of the contaminating bacteria.

B. Interaction of B. bacteriovorus 15143 and Blue-green algae.

B. bacteriovorus 15143 was originally added to a minimally contaminated culture of Oscillatoria. The results were startling in that within 30 min. all of the Oscillatoria ceased movement and never did regain it during observation which continued until the death of the Oscillatoria could be verified by microscopy or respirometry.

My laboratory found that active bdellovibrio cultures as well as culture supernatants (rendered cell free by centrifugation and filtration) were capable of breaking down the Oscillatoria. The disintegration caused by the enzymatic secretions of the bdellovibrios is extensive, resulting in the loss of all cytoplasmic cell contents and eventual dissolution of the cell wall (Fig. 2). In order that heterotrophic media be eliminated as a cause of this algal lysis, Oscillatoria cells were introduced into sterile tubes containing YP (yeast extract and peptone) medium or Nutrient Broth and observed for a period of days. In spite of the fact that these cultures became overgrown with contaminating bacteria, the condition of the algae remained normal in terms of motility and cellular organization.

The condition of the Oscillatoria was observed immediately after adding the bdellovibrios to determine if any immediate physiological damage not visible by microscopy was occurring. The results indicate that both respiration and photosynthesis were within normal values after 1.5 hours exposure to the bdellovibrios. Because it is very difficult to be sure of respiration and and photosynthesis data when contaminating bacteria are present, cultures of the blue-green algae Phormidium were employed as hosts for this experimentation with the bdellovibrios. The cultures of Phormidium are bacteria-free and allowed accurate measurement of oxygen uptake and evolution.

The data describing the interaction of B. bacteriovorus 15143 and P. luridum var. olivacea will be found described in detail in Appendix I (a manuscript entitled "Extracellular lysis of Phormidium luridum by Bdellovibrio bacteriovorus"). A summary of this data is as follows (Burnham and Stetak, 1972a):

Four day old cultures of blue-green algae, Oscillatoria sp. or Phormidium luridum, were interacted with 24 hr cultures of Bdellovibrio bacteriovorus ATCC #15143 in both solid and liquid environments. Phase contrast microscopic examination showed gradual structural changes in both species of algae until after 4 days of interaction only cell wall fragments remained. Corresponding to these structural changes there was a significant decrease in culture absorbance, a loss of measurable algal chlorophyll and an increase in protease activity in the culture. When photosynthesis was followed in the mixed culture of B. bacteriovorus and the bacterial-free Phormidium, a total inhibition of oxygen evolution occurred after 36 hours. Over 80 per cent of normal oxygen evolution was inhibited in less than 24 hours. When cell-free filtrates were used in place of viable B. bacteriovorus cells similar effects were observed. Light and electron microscopy have shown that active intracellular parasitism of viable algal cells by the bdellovibrios does not occur. When B. bacteriovorus or bdellovibrio culture filtrates were placed on lawns of Phormidium cells clear plaques were observed to form.

I have spent considerable time examining the inhibition of photosynthesis, as I feel sure this effect will be the key to isolation and purification of the lytic factor. Until recently, I have utilized a Gilson Respirometer for oxygen gas measurements in photosynthesis quantitation, which is slow, tedious, and subject to considerable error. Now we have developed a special chamber which employs a Yellow Springs Scientific oxygen electrode system coupled to amplifiers and a recorder to quantitate the changes in oxygen production. I believe that the chamber enclosing this system is unique, and I plan to publish its description and design to facilitate research of this nature in the future.

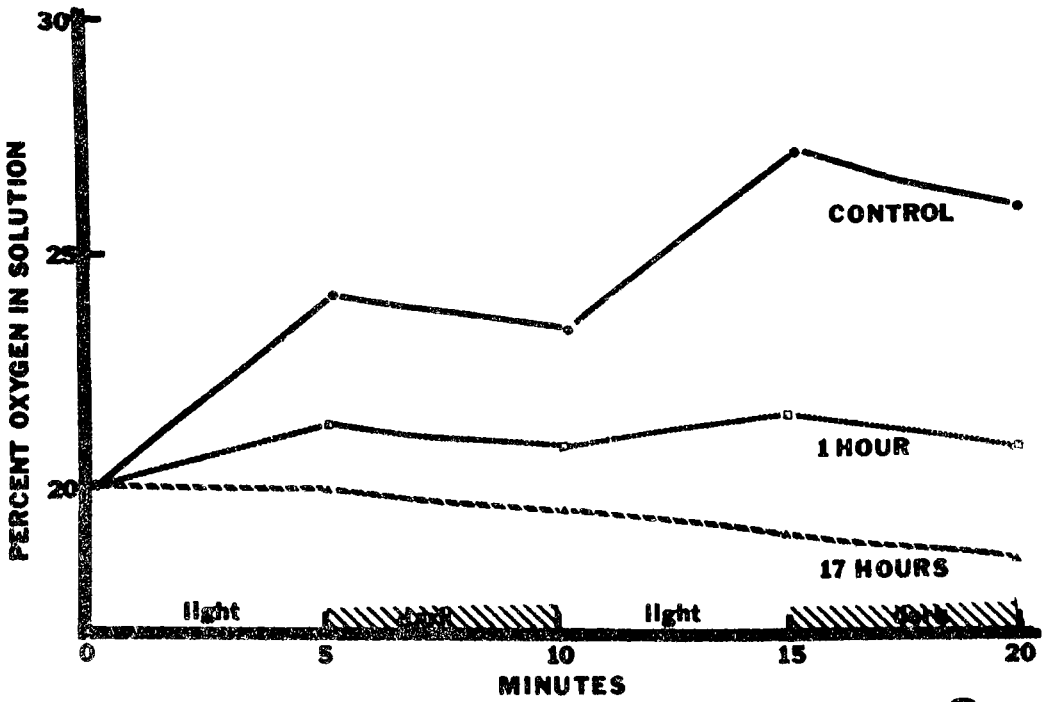
Basically, it is a square black chamber containing a double ring of fluorescent lights controlled by timing devices to allow adjustable alternate light and dark periods during which photosynthesis and respiration are measured. (Fig. 3) With this improved apparatus, we have found photo-synthesis can be easily and rapidly quantitated in experimental environments as tested against control blue-green algae. Equal portions (1.5 ml) of a 96 hr. P. luridum culture and bdellovibrio supernate were added to the electrode chamber and controlled at 30°C. Light is regulated so that alternate 5 min. light and dark cycles are recorded. The results of this are shown in Figure 3a. Note that the dark cycles of Phormidium mixed with the bdellovibrio supernate show equal respiration rates with the control Phormidium cells. The effect of the inhibitor is primarily in the oxygen evolving system as evidenced by Table 1 which tabulates that data shown in Figure 3a. It has been consistently found that photosynthesis is inhibited in P. luridum over 50% in less than 1 hour by addition of the bdellovibrio extract. Inhibition continues to increase with time until very little photosynthetic ability remains after 24 hours of exposure.

One of the major criticisms of Fiscal 1972 research results was that the soluble lytic factor described was probably a lysozyme-like muramidase as had been previously shown (Fackrell and Robinson, Canadian Journal of Microbiology 10:659, 1973) to be produced by some bdellovibrios. In order to see if this was true, we have tried approaching this question in several ways. Lysozyme was tested

Legend:

Figure 3a

This graph represents the values directly plotted from a Yellow Springs Instrument Co. oxygen analyzer containing suspensions of P. luridum and B. bacteriovorus 15143. The photosynthetic rate (oxygen output) of the control algal cells (during 5 min. light period) is over twice that of algal cells exposed to bdellovibrios for only 1 hr. The respiration values or oxygen uptake is approximately the same as the control cells regardless of length of bdellovibrio exposure. Table 1 converts these data to numerical rates.


30
25
20
PERCENT OXYGEN IN SOLUTION
CONTROL
1 HOUR
17 HOURS
light
light
0
5
10
15
20
MINUTES

3a

TABLE 1

EFFECT OF BDELLOVIBRIO SUPERNATANT FLUID
ON THE RATES OF P. LURIDUM PHOTOSYNTHESIS AND RESPIRATION

	Photosynthesis		Respiration	
	1 Hr.	17 Hr.	1 Hr.	17 Hr.
CONTROL	9.81*	11.40	1.67**	1.43
SUPERNATE	2.8	0.37	.80	1.02

*Numbers represent ul O_2 evolved / hr / ml Phormidium culture

**Numbers represent ul O_2 taken up / hr / ml Phormidium culture

against P. luridum cells to determine the lytic effect of the muramidase on the blue-green cell walls. Cell breakdown was observed in Phormidium in a 300 fc light environment after approximately 12 hours of exposure to a .05% lysozyme. The breakdown process did not resemble that caused by the soluble bdellovibrio lytic factor. The cells underwent severe distortion of the cell wall with cellular disruption soon following as can be seen in Figure 4. This is in marked contrast to the bdellovibrio factor which produces granulation, vacuolization, swelling, fragmentation and lysis. Cell distortion and breakdown were associated with a loss of photosynthetic ability with lysozyme treated cells whereas, with the bdellovibrio factor, photosynthesis was inhibited long before there were significant observable cellular morphological changes.

In addition the bdellovibrio supernatant fluid was heated to boiling for 20 minutes and then tested against P. luridum. A similar inhibition of photosynthesis but a slower cellular breakdown occurred when compared to the effect of non-heated controls. This data plus comparison with the lysozyme data above make it very unlikely that an enzyme such as a bacterial muramidase is the sole lytic agent. It should also be pointed out that unlike previously reported lytic enzymes of bdellovibrios, this soluble lytic factor affects viable healthy microorganisms rather than inactivated or heat-killed cells.

With our present culture technique of growing the bdellovibrios on E. coli substrates for 24 hours prior to exposing the blue-green algae, it was found that the bdellovibrio lytic factor could be diluted by a factor of 10 and still obtain an inhibitory response, although it takes longer for the effect to occur. Experiments with lytic factor dilutions indicate that 1 bdellovibrio cell is producing enough factor

to inhibit 75 P. luridum blue-green algae cells. It is hoped that we may further improve the production of this factor by altering the environmental conditions of culture.

Further studies were continued to resolve subcellular pathologic changes associated with P. luridum breakdown by the lytic factor. In order to guarantee that structural alterations were not artifacts of fixation, a series of comparisons were carried out utilizing a variety of aldehyde-osmium tetroxide mixtures in different buffers. The final results showed that 4% gluteraldehyde in a 2% osmium tetroxide contained in diluted veronal acetate buffer, i.e., a slight modification of a standard procedure for bacteria, provided superior results. Figure 5a shows a P. luridum cell fixed under those conditions. This fixation compares favorably with those reported by Allen (1968) and Lang (1968). The cell wall shows the typical "Gram-negative" envelope structure with a dense mucopeptide band of approximately 15 nm surrounding the cell. The photosynthetic system is comprised of double membranes concentrically arranged around the cell. These membranes are close together separated by a dense cytoplasm approximately 10 nm thick. Note that in these normal cells the paired membranes do not split into separate membranes. The center of the cell generally contains a fiber-like nucleoplasm mixed with a ribosomal cytoplasm. No distinct nucleoid is visible in any of the fixation methods. When bdellovibrios and bdellovibrio cell-free supernatant fluid were interacted with P. luridum (4 day old culture) the primary lesion was observed in the photosynthetic lamellae system (Fig. 5 b-f). The two membranes making up each lamellar layer split and the intermembranous space became filled with a rather homogeneous cytoplasmic matrix.

This matrix continued to increase in size until the membranes themselves became disorganized with general loss of distinguishable cytoplasm as seen in Fig. 5g,h. This is in direct contrast to the results found for lysozyme treated cells.

Ultrastructural examination of the algae during breakdown showed that little damage occurred to the cell-wall (Fig. 5b-d). Loss of the mucopeptide layer occurred only after several days incubation with the lytic factor. This is in direct contrast to the lysozyme treatment which resulted in a loss of algal mucopeptide in a matter of several hours (Fig. 6 b-d).

The data described above has allowed a better understanding of the action of the bdellovibrio lytic factor and how it is interacting with the host blue-green algal cells. It is quite apparent that rather than just a single factor being active, there are really two factors. One is a type of muramidase and the second is a unique inhibitor of blue-green algal photosynthesis. From this data I am able to proceed with the isolation of the heat resistant photosynthetic inhibitor and during this isolation, test each step by utilization of the oxygen analyzer chamber. Use of this facility should allow better sensitivity for fraction testing and allow a more rapid determination of the inhibitor's activity.

To date the data indicates that this material may have an excellent potential for blue-green algal control. Its stability in room temperature is excellent with no loss of activity occurring over three weeks; its stability to boiling indicates that it should be hearty enough to survive the environmental influences of nature; and its inhibitory activity indicates that it may effectively and irreversibly stop the growth of unwanted algae.

Further discussion of the results and implications of the investigation can be found in the "Discussion Section" of the Appendix I and in the "Application Section" of this report.

C. Effect of Selenium on Phormidium luridum

The ultrastructural breakdown of Phormidium by the B. bacteriovorus factors showed distinct alterations in the photosynthetic lamellar system. Another mechanism that was thought might equally produce alterations in the photosynthetic membrane organization was exposure to selenite ions. This data has been collected and published in a Masters Thesis entitled "The effect of selenium on the morphological and physiological properties of Phormidium luridum var. olivacea" and in a soon to be published article in the Journal of Phycology which is included in Appendix III of this report. A summary of this project which was presented to the American Society of Microbiology Annual Meeting in Miami Beach, April, 1973 is included below:

Bacterial-free cultures of the blue-green algae Phormidium luridum var. olivacea were treated with 10^{-6}M to 10^{-2}M sodium selenite under conditions for normally maximal cell growth. In contrast to increasing culture turbidity of control and 10^{-6}M selenite cultures, the turbidities of the other selenite cultures declined in direct proportion to time and increasing selenite concentrations. Chlorophyll quantitation revealed similar results. Photosynthetic oxygen production was inhibited within 24 hour in all cultures (10^{-5}M to 10^{-2}M) except the control and 10^{-6}M selenite cultures. Light microscopy revealed red granules of reduced selenium associated with the surface of the P. luridum and after 72 hr. many of the red granules were observed free in the culture medium. Other structural changes observed

included the development of intracellular and intercellular spaces, spheroplast formation and gradual cell lysis. Electron microscopy also revealed a distortion of the photosynthetic lamellae in the selenite-treated cells that is generally observed only in extremely old P. luridum. Protein analyses confirmed the rates of cellular breakdown.

It is significant to note that Phormidium cells respond to the adverse stimuli in a very similar manner; i.e., loss of photosynthetic ability and severe distortion of the photosynthetic membrane organization. Preliminary evidence (unpublished) indicates that the aging process of P. luridum results in similar patterns of structural disorganization. If we are to develop biological control systems for the destruction of masses of blue-green algal populations, it is important that the pathological changes caused by these agents be understood.

D. Technique Development

Although several techniques have been devised in order to carry out this project (i.e. algal medium for parasite isolation) one has been found significant enough to be passed to all investigators. Appendix II of this report describes the method we developed during this project for the enumeration of the Phormidium cells contained in the cultures being exposed to the bdellovibrios. This method of counting cells in microbial filaments makes a tedious job much easier and more accurate as well.

E. Application of Research Results

Bacteria have recently been shown to lyse several species of blue-green algae by various mechanisms. My recent work (Burnham and Stetak, J. of Bacteriology, 1973 with bdellovibrios and that of Granhall and

Berg (1972) have shown that it is possible to use bacteria in liquid environments to lyse and control algal populations.

It is particularly significant that cell-free extracts of bdellovibrio cultures are able to cause lysis of two blue-green algal species. There have been no other reports of bdellovibrio enzymes being employed on a cell-free basis to affect living microorganisms. The fact that the lytic factor is cell-free will facilitate its location and purification with the eventual possibility that a small sample of it can be added to natural water resources to control algal populations, particularly where algae tend to exist in very high numbers, such as blooms, rock, wood and other substrates located in the water. The ability to add a natural substance to the water in reasonably small quantities offers unlimited possibilities to boaters, marinas, small reservoirs, farm ponds and watering sources, natural and tropical fish fanciers and many others who are troubled with algal overgrowth problems mentioned above.

It is important that in addition to the investigation of the lytic process, the proposal should determine the nature of the inhibitor of photosynthesis (Burnham and Stetak, J. of Bacteriology, 1973) produced by the bdellovibrios. I have only observed one other account of photosynthesis being inhibited by another living organism on a direct interaction basis. This was a recent account of the inhibition of photosynthesis of the green algal genus, Volvox, by another colonial green algal genus, Pandorina (Harris, 1971). Inhibition of blue-green photosynthesis by the bdellovibrios is particularly significant again in regard to controlling algal populations. It may be possible to find concentrations of the secreted factors from bdellovibrios that

will only inhibit the photosynthetic capabilities of an algal population without lysing them, thereby, achieving the maximum in algal control.

It should be reemphasized that this research deals with understanding a host-parasite relationship in order to control population of algae. This investigation should provide insight into biological control mechanisms--not only as to how algal populations can be biologically controlled, but also how parasitic microorganisms are involved in natural control of aquatic organisms. The algal-bacterial interaction that has been described should not be unique to the laboratory and may play an important role in natural ecosystems. In this regard the local environment (i.e., Maumee River and Maumee Bay in Western Lake Erie) offers an ideal opportunity and reason for this research. Algal blooms are constantly being publicized as creating health, economic and aesthetic problems. The public concern in this area dictates that possible solutions should be sought immediately.

It is also important to consider the nature of the host organisms employed in this study. The blue-green algae occupy a unique phylogenic and evolutionary niche in nature by being taxonomically placed between the bacteria and the higher plant forms; they have a unique motility which is not understood; they have a distinct structural organization; they are photosynthetic; they are autotrophic; they are extremely common to all water resources; and, they are the causative agents of a variety of important problems. The above characteristics make these organisms fascinating to work with and also economically important organisms to understand.

It has been well documented that both bdellovibrios and blue-green algae are very common in natural water habitats. This, plus evidence that bdellovibrios directly attack Phormidium and are capable of lysing a species of blue-green algae in an agitated liquid environment, suggests that these bacteria may play a role in the control of blue-green algal populations in their natural ecosystems. The natural tendency of B. bacteriovorus to interact with other microorganisms makes the relationship indicated here a more likely phenomenon in nature.

F. Work Remaining

Of course, the long range goal of this project remains to be achieved; i.e., to develop an effective means to utilize these bacteria to eliminate and control blue-green algal populations in natural water resources. It is important to realize, however, that our recent findings indicate that such a goal is evermore realistic in scope. The objectives of the next phase of this project can be listed as follows:

1. To isolate and identify the photosynthetic inhibitor produced by B. bacteriovorus ATCC 15143.
2. To isolate and identify the cell wall lytic factor produced by B. bacteriovorus ATCC 15143.
3. To develop either facultative or host independent algal parasites, thereby, eliminating the present need for bacterial host intermediates.
4. To examine the influence of environmental conditions in the production of the anti-algal factor and its interaction with the blue-green algal cells.
5. To determine the maximum effectiveness of the anti-algal factor produced B. bacteriovorus ATCC 15143 in both eliminating and controlling blue-green algal populations.

This project is going to continue with the support of the Title I Matching Grant from the OWRR U.S. Department of Interior for FY 1974 through 1976. As stated in that grant application, this period will hopefully find this agent a practical, useful, and significant factor in algal control.

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PLATE 1

LEGEND:

Figure 1 - Macrophotograph of bacterial isolate A12 lysing a lawn of Chlorococcum on an agar surface.

Figure 2a - Phase contrast micrograph of an Oscillatoria trichome immediately after introduction of bdellovibrio cells. The trichome appears identical to untreated healthy cells.

Figure 2b - Phase contrast micrograph of an Oscillatoria trichome showing the empty cells commonly found after approximately 96 hr. of exposure to Bdellovibrio bacteriovorus 15143 culture.

Figure 2c - Phase contrast micrograph of a completely disrupted Oscillatoria trichome typically seen after 96 hr. exposure to the bdellovibrio. The Oscillatoria cell walls appear broken and disorganized.

Figure 3 - Photograph of an oxygen analyzer chamber constructed by Mr. Tom Stetak and myself to allow consecutive light-dark, photosynthesis and respiration measurements. The chamber is light-tight and contains a Yellow Springs Instrument Co. measurement chamber with two oxygen electrodes, a double ring of fluorescent light giving a foot candle intensity of 1,000, and a timing mechanism to control cycling of the lights. This apparatus is connected to two amplifiers and then to a double pen recorder.

Figure 4 - Phase contrast micrograph of P. luridum cells 12 hr. after exposure to 0.05% lysozyme in algal broth. The normally long trichome is completely broken into single cells. Cellular damage can be seen to include complete disruption and severe collapse of the algal protoplast.

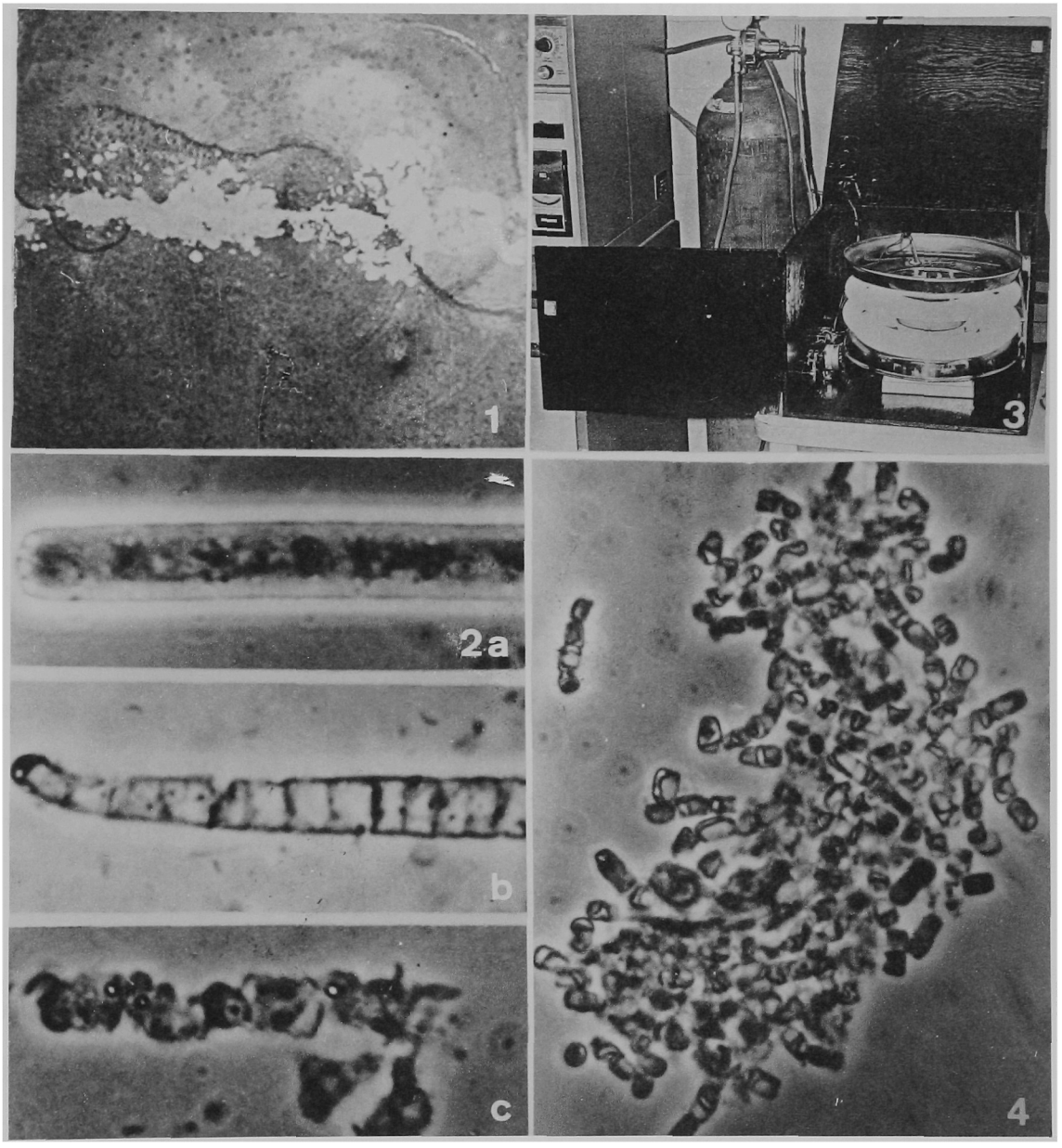

1
3
2a
b
c
4

PLATE II

Legend:

Figure 5a. This section of P. luridum grown in algae broth for 96 hr. This cell represents the ultrastructure normally seen in Phormidium cells with the gram negative cell wall structure and a dense ribosomal cytoplasm separated by concentric double membranes comprising the photosynthetic system. Note that the photosynthetic membrane pairs are normally only separated by approximately 10 nm. X65,000.

Figure 5b. This section of P. luridum interacted with B. bacteriovorus culture supernatant for 14 hr. Intermembraneous spaces are forming typical between the paired photosynthetic menbranes. The protoplast-like bodies formed by the enclosure of a portion of the cytoplasm by a photosynthetic menbrane is also seen. X42,000.

Figure 5c. This section of P. luridum mixed with a bdellovibrio culture for 24 hr. This micrograph illustrates that the mucopeptide layer of the Phormidium cell wall has not been degraded nor is there any appreciable loss of cell shape. The intermembraneous spaces are clearly seen as is a bdellovibrio cell over the upper edge of the algal cell envelope. X70,000.

Figure 5d. This section of P. luridum mixed with bdellovibrio culture supernatant for 24 hr. Protoplast-like bodies dominate the internal area of the algal protoplast. The mucopeptide layer of the algal wall is still apparent. X48,000.

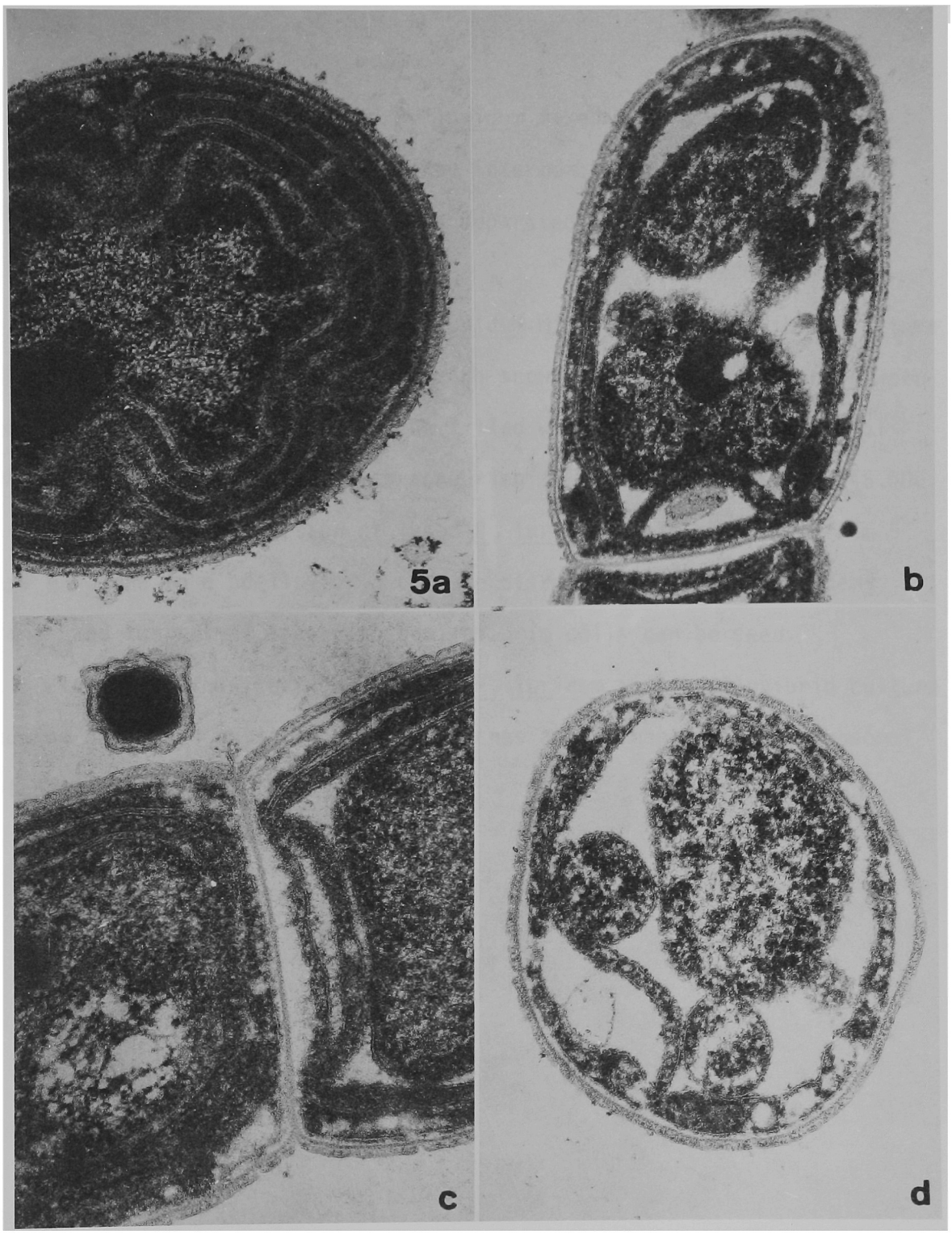

5a
b
c
d

PLATE III

Legend:

Figure 5e. This section of P. luridum mixed with bdellovibrio culture supernatant for 24 hr. Large intermembraneous spaces fill the cell as the photosynthetic system is separated along each membrane pair. X48,000.

Figure 5f. This section of P. luridum mixed with bdellovibrio culture supernatant for 48 hr. This micrograph shows that the separation between the photosynthetic membranes can be filled with a homogeneous material considerably distinct from holes appearing in poorly fixed cells. X46,000.

Figure 5g. This section of lysed P. luridum cells resulting from 5 days exposure to a bdellovibrio culture disrupted photosynthetic membranes and two normal appearing bdellovibrio cells can be seen.

Figure 5h. This section of lysed P. luridum in a bdellovibrio culture showing disrupted photosynthetic membranes and remnants of the ribosomal cytoplasm.

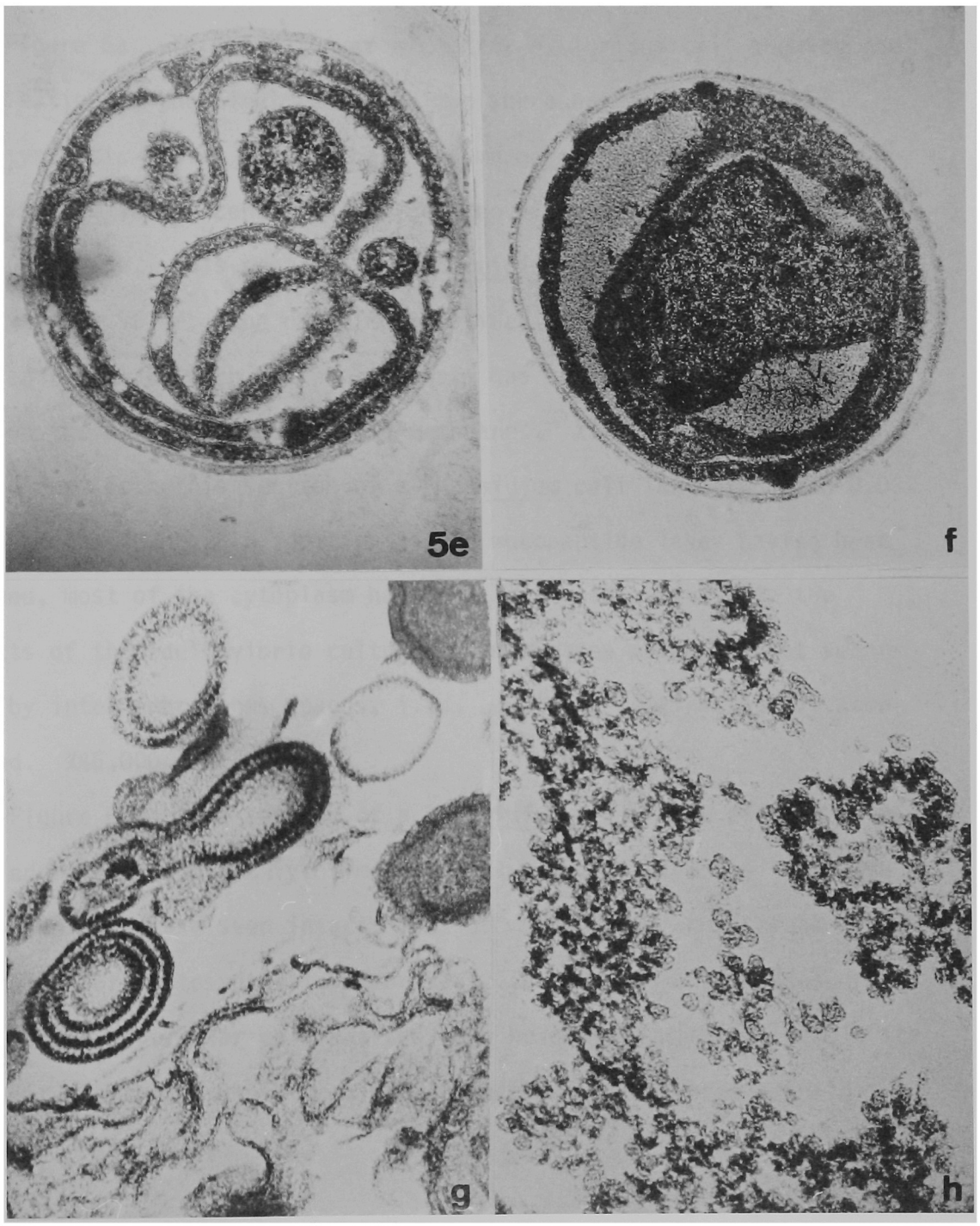

5e
f
g
h

PLATE IV

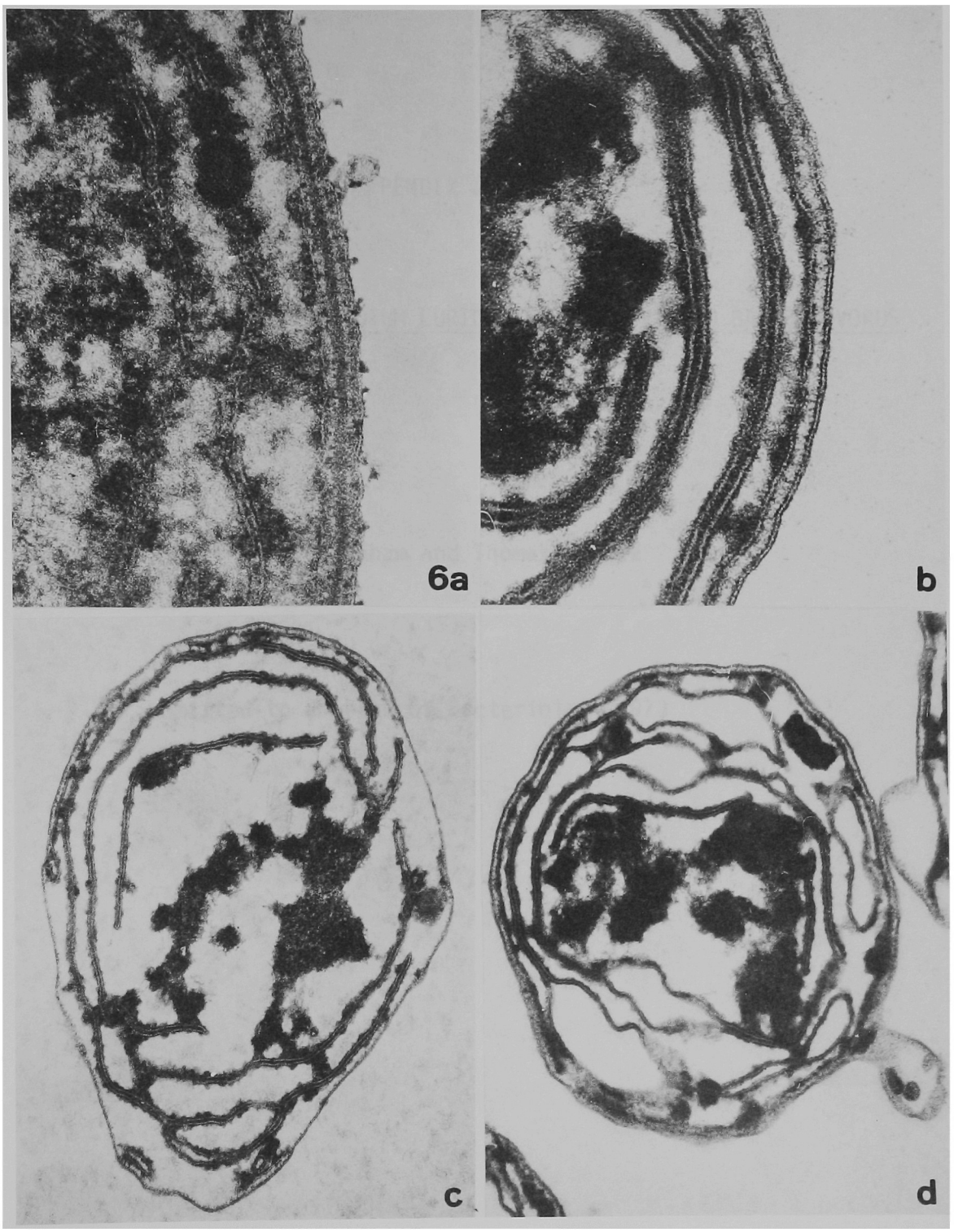
Legend:

Figure 6a. This section of a control P. luridum cell showing the organization of the algal cell envelope surrounding the cytoplasm photosynthetic system. Note the dense mucopeptide layer of approximately 15 nm in the center of the cell envelope. X120,000.'

Figure 6b. This section of a Phormidium cell incubated with 0.05% lysozyme for 96 hr. The dense mucopeptide layer is missing from the cell envelope and much of the cytoplasm has been lost from the area between the paired photosynthetic membranes. X140,000.

Figure 6c. This section of a Phormidium cell incubated with 0.05% lysozyme for 48 hr. In addition to the mucopeptide layer having been removed, most of the cytoplasm has been lost. In contrast to the effects of the bdellovibrio culture the membranes pairs are not separated by intermembraneous spaces, i.e., each pair remains tightly associated. X46,000.

Figure 6d. This section of a Phormidium cell shows effects of 96 hr. incubation in 0.05% lysozyme. Cellular organization is lost with almost no cytoplasm seen intact; loss of mucopeptide and gradual disorganization of photosynthetic membranes. Intermembraneous spaces are seen in the particular cell but they are never seen with the regularity of their occurrence when the algae are exposed to the bdellovibrio cultures. X42,000.


6a
b
c
d

APPENDIX I

EXTRACELLULAR LYSIS OF *PHORMIDIUM LURIDUM* BY *BDELLOVIBRIO BACTERIOVORUS*

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EXTRACELLULAR LYSIS OF *PHORMIDIUM LURIDUM* BY *BDELLOVIBRIO BACTERIOVORUS*[1]

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[1] A portion of this paper was presented at the Annual Meeting of the American Society for Microbiology - Philadelphia - April 1972.

ABSTRACT

When either cells of Bdellovibrio bacteriovorus strain 15143 or a heat resistant lytic factor derived from these cells are added to viable cultures of Phormidium luridum var. olivacea all of the algal cells underwent gradual lysis. This effect was obtained with an initial bdellovibrio to algae cell ratio of 1 to 7.5. When P. luridum was mixed with the bdellovibrio cultures the algal chlorophyll content showed an 8 to 10 fold decrease. Light microscopy revealed that non-refractile granules formed in the P. luridum within 2 hr after adding the bdellovibrio lytic factor. Subsequent morphological changes included the development of intracellular and intercellular spaces, spheroplast formation and finally complete lysis of the blue-green algal cells.

INTRODUCTION

The ability of Bdellovibrio bacteriovorus to infect, to multiply within, and then to lyse a variety of Gram-positive and Gram-negative bacteria is well documented (2, 4, 11, 19, 20, 21, 23). Because of the many biochemical and structural similarities between bacteria and blue-green algae (5, 13); the susceptibility of blue-green algae to lysis by other bacteria (9, 16, 21) and phage (17); and the lysis of certain green algal species by a bdellovibrio-like bacterium (15); it seems reasonable to expect that the bdellovibrios would produce similar effects on the blue-green algae.

This report is the first to describe a lytic effect of bdellovibrios on blue-green algae. It also indicates that an extracellular secretion of bdellovibrios can affect viable microorganisms in contrast to previous reports showing the affect of secretions on non-viable cells (20, 21). The physiological and morphological details of the extracellular bdellovibrio/blue-green algae interaction are presented in this paper.

MATERIALS AND METHODS

Organisms. The blue-green algal strain used in this study, Phormidium luridum var. olivacea (Species #426), was obtained from the Culture Collection of Algae at Indiana University. The B. bacteriovorus (strain #15143) and the host bacterium, Escherichia coli (strain #15144) were obtained from the American Type Culture Collection.

Media and cultural conditions. Routinely, 100 ml. of Difco algae broth (designated AB) contained in 500 ml Erlenmeyer flasks was inoculated with a bacterial-free culture of P. luridum and placed on a reciprocal shaker at 25C. The light intensity for the growth of P. luridum was 300 foot candles. These cultures were allowed to develop for 96 hr before being mixed with the bdellovibrios.

Because B. bacteriovorus strain 15143 is an obligate parasite, it was routinely maintained on cultures of E. coli contained in yeast extract - peptone (Difco) medium (designated YP) as previously described (2). For experiments involving mixtures of algae and bacteria, a 5 per cent inoculum of B. bacteriovorus was introduced into a 24 hr culture of E. coli and allowed to develop for 24 hr at 30C on a rotary shaker before being added to the P. luridum cultures.

Bacterial-algal interaction. Twenty-four hr cultures of bdellovibrios were always added to an equal volume of 96 hr cultures of P. luridum for direct interactions (designated P + Bd). Generally 50 ml of the 24 hr culture of bdellovibrios were added to 50 ml of the 96 hr culture of P. luridum contained in a 500 ml side arm Erlenmeyer flask (Bellco). The flasks were then incubated at 25C on a reciprocal shaker under 300 fc. Control cultures employed in this study were: (a) 50 ml of a 96 hr culture of P. luridum and 50 ml of sterile AB medium; (designated P + AB); and (b) 50 ml of a 96 hr culture of P. luridum and 50 ml of sterile YP medium (designated P + YP). Cell-free supernatants were prepared by centrifuging 24 hr cultures of

the bdellovibrios at 10,000 x g for 20 min, and carefully decanting the supernatant fluid. This fluid was then filtered through a 0.22 um pore filter (Millipore Corp., Bedford, Mass.) and designated P + S.

Light Microscopy. Samples were observed throughout the experiments by phase contrast microscopy using a Zeiss Universal Photomicroscope fitted with a Nikon automatic exposure meter and camera. All pictures were taken using the Zeiss microscope flash attachment. Samples were not washed and were not chemically fixed or centrifuged prior to examination.

Assay procedures. Culture turbidity was determined spectrophotometrically using side arm flasks (Bellco) in a Coleman Junior Model II Spectrophotometer at 610 nm.

Chlorophyll was extracted from the Phormidium after centrifuging them out of the experimental culture at 10,000 x g for 10 min. The cells were suspended in a 2:7 acetone: methanol mixture (3, 14) and then shaken at 4C for 4 hr. This mixture was then centrifuged to remove cellular material. Chlorophyll was quantitated at 660 nm on a Gilford Model 2400 Recording Spectrophotometer as described by Parsons and Strickland (16). The experimental culture supernatant fluid was examined by scanning spectrophotometry (Beckman Acta V) for pigment absorption in order to guarantee complete chlorophyll measurement. Reextraction of cell pellets did not yield measureable chlorophyll.

Photosynthesis was determined in a Gilson Respirometer at 30°C with a light intensity of 1200 fc. In order to standardize conditions of photosynthetic measurement P. luridum cells were always centrifuged out of their culture supernatant fluid and resuspended in bicarbonate solution #9 (24).

Respiration was determined by incubation of P. luridum/bdellovibrio mixture in a Gilson Respirometer at 30°C using 20% KOH in the CO_2 trap (24).

The number of B. bacteriovorus per ml was determined by the plaque technique previously described (4). Phormidium cells were quantitated directly by dry weight following two washes to remove contaminating salts, and by cell counts utilizing a Petroff-Hauser Counting Chamber.

Protease activity of cell cultures and supernatant fluids were determined using Azocoll (Calbiochem) as previously described (4).

RESULTS

The combination of a 24 hr culture of B. bacteriovorus grown on E. coli host cells and a 96 hr culture of P. luridum showed that the bdellovibrios gradually lysed the blue-green algae in 2 to 4 days. The bdellovibrios were microscopically observed to attach and detach at random, but they did not permanently attach to or penetrate the P. luridum cell wall. This observation has been confirmed by subsequent electron microscopic examination which revealed no direct penetration into the blue-green cells by the bdellovibrios.

When the P. luridum and the B. bacteriovorus cells were quantitated immediately after being mixed together in the experimental flask, it was found that there were an average of 6×10^8 bdellovibrios per ml and 4.5×10^9 P. luridum cells per ml. Thus there was an average initial ratio of 1 bdellovibrio cell for every 7.5 P. luridum cells in the mixed culture.

The gradual loss of absorbance, when the P. luridum was added to the viable bdellovibrio culture or to the bdellovibrio culture supernatant, is shown in Fig. 1. The difference in absorbance between the P + Bd and P + S cultures can be attributed to the turbidity added by the B. bacteriovorus cells and residual E. coli cells from the original bdellovibrio culture. When P. luridum interacted either with the bdellovibrio culture or with the culture supernatant fluid, an average loss of 50 per cent of initial absorbance was observed over a period of 1 week. A similar effect occurred when the cell-free bdellovibrio supernatant was boiled for 20 minutes prior to addition to the algal culture. P. luridum cultures gradually increased in turbidity when they were inoculated into fresh algae broth, and an even more marked increase occurred when the organic YP medium was substituted for the AB medium, (Fig. 1).

When the cell-free supernatant fluid from a 24 hr culture of the bdellovibrio

host organism E. coli, was added to P. luridum no effect on the blue-green algae was noted. Experiments employing ultrasonically broken E. coli as the test agent also showed no affect upon the P. luridum.

To determine the effect that bdellovibrio cells had on P. luridum, bdellovibrios were centrifuged out of the culture and resuspended in 100 ml of sterile YP medium or sterile AB medium (approximately 5 x 10^8 cells per ml). The bdellovibrios were subsequently added to an equal volume of a 96 hr culture of P. luridum. When suspended in YP, the bdellovibrios exhibited a lytic activity resulting in a 25 per cent reduction of mixed culture turbidity, however, when bdellovibrios were resuspended in AB medium, no observable effect on the blue-green algae was recorded. Subsequent observations indicated that when washed bdellovibrios were added to sterile AB medium they rapidly lost their motillity and formed spheroplasts in less than 24 hr. This morphological change explained why no lysis of P. luridum occurred when the washed bdellovibrios were added to the algal culture.

When cultures of P. luridum mixed with bdellovibrios were examined periodically for 7 days a marked pigment change became evident as a bright green algal culture was turned colorless and translucent. To quantitate this pigment change a chlorophyll extraction was carried out on both control and experimental cultures, the results are shown in Fig. 2. The amount of chlorophyll per ml of P. luridum control cultures in AB and YP media showed distinct increases over 6 days. The chlorophyll in the P + Bd culture decreased approximately 6.5 fold over 6 days while in the P + S culture the loss in chlorophyll per ml was approximately 10.5 fold. In all cases the experimental culture supernatant fluid contained no measurable chlorophyll.

Fig. 3 illustrates the rapid loss of photosynthetic ability of the algae exposed to both bdellovibrio cultures (P + Bd) and cell-free supernatant fluids (P + S). More than 50% inhibition occurred in less than 24 hours with 95% inhibi-

tion occurring within 48 hr. In marked contrast, was the rapidly increasing oxygen production by the P. luridum in AB and YP control media.

In order to determine if algal cell respiration correlated with photosynthesis values oxygen uptake was measured. P. luridum was tested only in the bdellovibrio cell-free supernatant in order to eliminate any possibility of measuring the bdellovibrio's respiration. No inhibition of respiration occurred for 3 days. Thereafter respiration levels decreased steadily with approximately 80% inhibition evident by the end of 7 days.

The loss of culture turbidity, the reduction in chlorophyll levels, and the loss of photosynthetic ability in both the P + Bd and P + S cultures, were partially explained by phase contrast light microscope observations as shown in Fig. 4 and 5. In Fig. 4a and b, P. luridum from control cultures of AB medium, can be seen to be unchanged after 2, and 160 hrs. Fig. 4c and d show that after 2 and 96 hr respectively, control cells grown in 50 per cent YP (P + YP) do not show any aigns of lysis or ill effect from the organic medium. The P. luridum cells in P + YP culture are longer than the control cells in the AB medium. Transparent areas can be seen in the nucleoid region in both the P + AB and Y + YP cultures (Fig. 4a, c) but these do not appear to be the same as the refractile yellowish granules seen during P. luridum breakdown in Fig. 5.

Fig. 4e to h show P. luridum from a P + Bd culture. Residual E. coli and B. bacteriovorus can also be seen in these micrographs. Normally there are only a few bdellovibrios that can be seen attached to the P. luridum cells. Interestingly, in most cultures of P. luridum infected with viable bdellovibrios, there are a few trichomes which appear to attract the bdellovibrios to attach to their surface. This results in the trichomes early lysis. No initial difference could be observed micro-

scopically between these few susceptible blue-greens and the remainder of the P. luridum trichomes. An example of this is shown in the micrograph Fig. 4e which was taken 6 hr after addition of the bdellovibrios. The P. luridum cells in the filament are covered with bdellovibrios and each algal cell shows evidence of lysis. No explanation is presently available for this phenomenon.

In the course of the interaction with the bdellovibrio culture various large intracellular spaces (Fig. 4f) are formed in areas adjacent to the algal cell wall. These spaces appear to be formed by contraction of the cell protoplast leaving a gap between the cytoplasmic membrane and the cell wall. Fig. 4g and h represent the final stages of P. luridum breakdown with complete disintegration of the blue-green trichome into individual cells with each cell showing evidence of cellular lysis.

The gradual breakdown and lysis of P. luridum in combination with the cell-free bdellovibrio supernatant is shown in Fig. 5. Granulation occurs soon after the algae are added to the bdellovibrio supernatant fluid as can be seen in Fig. 5b, c. The granules become refractile under phase contrast microscopy (Fig. 5e and i) and are of a yellow color when observed with bright field microscopy. The same intracellular spaces develop as in the P + Bd culture with these spaces occasionally occupying the majority of the cell (Fig. 5d, g, h). Although some cells are barrel shaped in both control and mixed cultures (Fig. 5d), most of the cells in the trichome retain their original shape for about 4 days in P + S cultures. They then usually round up (Fig. 5i), fragment and lyse (Fig. 5j). In addition to granulation one of the earliest signs of cellular breakdown is the fragmentation of the trichome (Fig. 5e, f, i) where individual cells retain their original shape but separate from adjacent cells.

When P. luridum in P + S cultures were incubated in the dark, they became granulated, separated, eventaully rounded up and lysed in less than 48 hr (Fig. 5k, l).

Both viable bdellovibrios and bdellovibrio supernatant fluid cause plaques to form on the lawns of P. luridum a 1.5 per cent AB agar. Plaque formation was variable, in contrast to the very regular and predictable lysis in liquid systems, as sometimes lysis occurred 5 days and in other experiments did not occur after two weeks. Microscopic examination showed the area immediately along the edge of the plaques to be an olive-brown color suggesting that the blue-green algae were about to lyse.

In looking for the lytic factor contained in culture supernatant fluids of B. bacteriovorus ATCC 15143, we examined the amount of protease in the supernatants of P + AB, P + YP, P + Bd and P + S cultures (Fig. 6). The P + Bd and P + S cultures had high levels of protease activity initially, but these levels dropped over 80 per cent in 48 hr, and by 72 hr activity in each culture had become immeasurable. The initial loss of protease activity in the P + Bd and P + S cultures was explained by observing a similar protease reduction in supernate from 24 hr bdellovibrio cultures (70% loss of activity in 24 to 48 hr). Unexpectedly, the protease activity began to increase after 3 days in both the P + Bd and P + S cultures. The later increases in protease activity is believed to be associated with lysis of the P. luridum. Throughout the entire experiment P + AB and P + YP controls possessed low levels of protease activity.

DISCUSSION

B. bacteriovorus has been known to infect and lyse various viable bacterial host cells, but only by means of intracellular attack. This is the first report of bdellovibrios lysing viable microorganisms extracellularly or affecting species of blue-green algae. The specificity of action of *B. bacteriovorus* ATCC 15143 in lysing blue-green algae has not been determined, although recent experiments indicate that an *Oscillatoria sp.* (Carolina Biological Supply Co.) is readily lysed, while *Anacystis nidulans* (Meyer's Strain) is not affected (Unpublished data).

Production of bacteriolytic and algal lytic enzymes is well documented for a variety of bacteria (8, 18, 22). Extracellular enzymes secreted by members of the Myxobacteriales (18, 22) are capable of lysing algal populations. Direct cell to cell contact is necessary in these systems as algal lysis in liquid culture is absent if the culture is agitated (18), thereby, probably reducing the localized concentration of enzyme. The extracellular protease concentration in *B. bacteriovorus* 15143 was measured and found to be significant. Whether this enzyme is participating in the lysis of the *P. luridum*, is not clear. Previous reports of bacteriolytic enzymes have involved protease activity very closely associated with mucopeptidase activity (5, 6, 7, 12), therefore, it is felt that although the bdellovibrio protease may not be the responsible agent for algal lysis, its presence can be used as an indicator for the lytic factor.

It is probable that a mucopeptidase is responsible for this algal lysis because: a mucopeptide has been shown to account for over half of the cell wall dry weight of a *Phormidium* species (5); and bdellovibrio species are known to possess a muramidase (21). In addition, a recent report of an antibiotic-like lysis of blue-green algal cells by *Cellvibrio* filtrates closely resembled the physical lysis of *P. luridum* described above. However, the lysis did not occur in the dark as did the

bdellovibrio caused lysis of P. luridum (10). Because of this difference, it is not likely that a similar antibiotic-like substance is being produced by the bdellovibrios.

Our light microscopic observations showed that shortly after the P. luridum had been mixed with the bdellovibrio supernatant fluid, structural changes, such as the gradual formation of granules and intracellular spaces (Fig. 5) occurred. As the presence of the bdellovibrio cells is not necessary, the lytic factor has to be a soluble substance released by the bdellovibrios during their growth in the host culture of E. coli. It was also shown that this substance does not originate from independently grown E. coli.

It has been shown that bdellovibrio enzymes in a culture supernatant fluid were capable of lysing heat killed Pseudomonas fluorescens, Bacillus megaterium and Spirillum serpens cells, but they could not lyse viable cells of the same species (7, 20). Shilo (20) suggested that the heat treatment sufficiently damaged layers of the bacterial wall to allow the protease access to the protein components of the wall and cytoplasm. Similarly, the soluble factor secreted by the bdellovibrios could indirectly produce a weakened cell wall as a result of interference with normal algal metabolism, i.e., evidenced by loss of photosynthetic ability and changes in cell morphology. The damaged envelope might then allow penetration of the lytic enzymes to complete the lysis of both the algal wall and cellular contents. In addition to being capable of affecting intact viable cells, the lytic factor is extremely heat resistant. This makes it distinctly unique from previous bdellovibrio peptidase and protease (7, 8). Studies are presently under way to identify the lytic factors in the bdellovibrio supernatant.

ACKNOWLEDGMENTS

This investigation was supported in part by a grant from the Owens-Illinois Corporation, Toledo, Ohio; an Ohio Water Resources Allotment Grant (#A-025-OHIO); and a General Research Support Grant from the National Institutes of Health (#5-S01-RR05700-02).

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LEGENDS TO FIGURES

Fig. 1. Effect of B. bacteriovorus 15143 on growth of P. luridum: Symbols: Four-day old culture of P. luridum plus an equal volume of: (a) AB medium (P + AB); (b) YP medium (P + YP); (c) 24 hr culture of B. bacteriovorus (P + Bd); (d) cell-free supernatant fluid from a 24 hr culture of B. bacteriovorus (P + S).

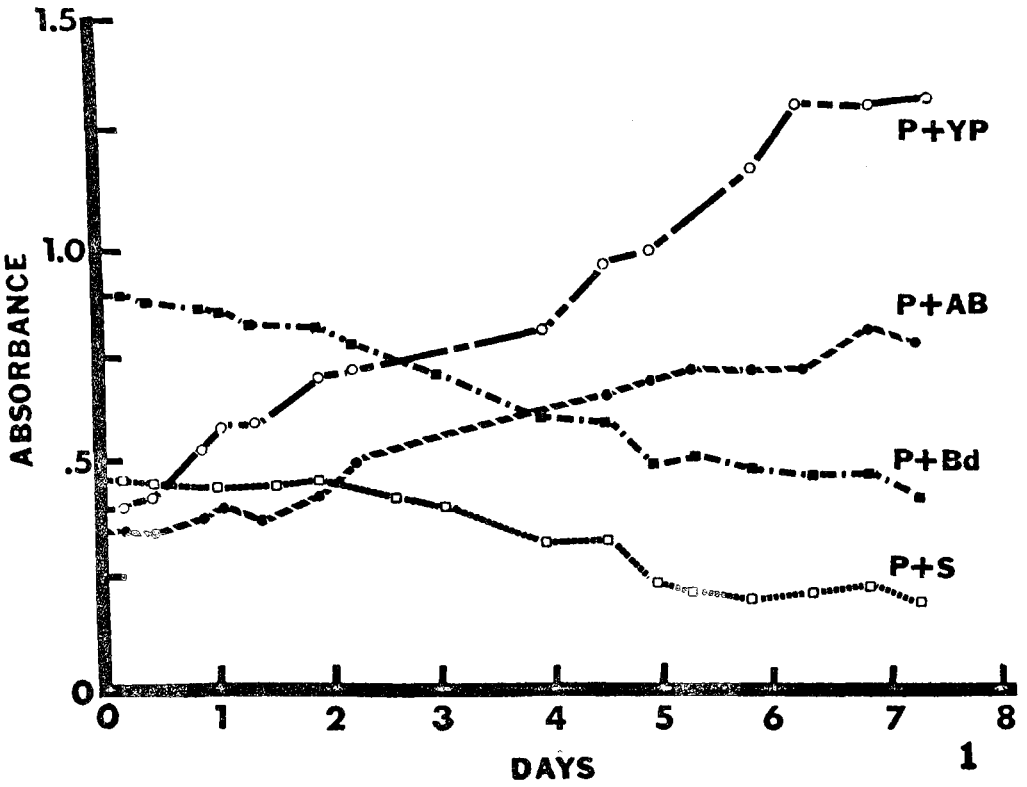
Fig. 2. Comparison of the chlorophyll content of P. luridum cultures mixed with an equal volume of: (a) AB medium (P + AB); (b) YP medium (P + YP); (c) 24 hr culture of B. bacteriovorus 15143 (P + Bd); (d) cell-free supernatant fluid from a 24 hr culture of B. bacteriovorus 15143 (P + S). Each point on all curves represents absorbance per ml culture supernatant fluid.

Fig. 3. Curve showing photosynthetic activity of P. luridum cultures as effected by bdellovibrio cells and cell-free supernatant fluid. Designations as described in Fig. 2.

Fig. 4. Phase contrast micrographs of P. luridum cells: (a) after 2 hr in P + AB culture (for description of letter designations see legend of Fig. 5); (b) after 160 hr in P + AB culture; (c) after 2 hr in P + YP culture; (d) after 120 hr in P + YP culture. (Arrow indicates small non-refractile granules); (e) after 6 hr in P + Bd culture. The individual P. luridum cells appear to be disintegrating, forming spheroplasts (S) and lysing (arrows); (f) after 40 hr in P + Bd culture (intracellular spaces are beginning to develop (IS); (g) after 72 hr in P + Bd culture (complete lysis has occurred with large clumps of broken cells usually surrounded by increasing numbers of residual E. coli; (h) after 190 hr in P + Bd culture. Bar represents 1 micrometer. All micrographs are the same magnification (X2500).

Fig. 5. Phase contrast micrographs of P. luridum cells showing the effect of an equal volume of cell-free supernatant from a 24 hr culture of B. bacteriovorus 15143 (P + S); (a, b) after 2 hr there is a small increase in granulation (G); (c) after 16 hr granulation is more pronounced (G); (d) after 48 hr large intracellular spaces develop (IS). (The barrel-shaped appearance of the P. luridum is not abnormal as it is seen as well as the individual cells in the filament beginning to separate (arrows); (f) after 109 hr granules (G) large intracellular spaces (S), and very short filaments are common; (g,h,i,j) after 120 hr. In addition to granules (G) intracellular spaces (S) and increasing amounts of cell separation (arrows), the P. luridum cells are forming into spheroplasts. Many of the P. luridum cells at this stage have begun to lyse as well (L); (k, l) after 48 hr of culture interaction in the dark. Complete cell separation spheroplast formation and cell lysis has resulted from the bdellovibrio enzyme. Amounts of cell separation (arrows), the P. luridum cells at this stage have begun to lyse as well (L); (k, l) after 48 hr of culture interaction in dark. Complete cell separation, spheroplast formation and cell lysis has resulted from the bdellovibrio enzyme. Bar represents 1 micrometer. All micrographs are the same magnification (X2500).

Fig. 6. Comparison of protease content of P. luridum cultures mixed with an equal volume of (a) P + AB; (b) P + YP; (c) P + Bd; (d) P + S. (For description of letter designations see legend of Fig. 5). Protease levels (absorbance per ml culture supernatant) is expressed relative to the level of protease contained in the P + Bd culture supernatant at zero time. Azocoll controls (no supernatant material) were set at zero.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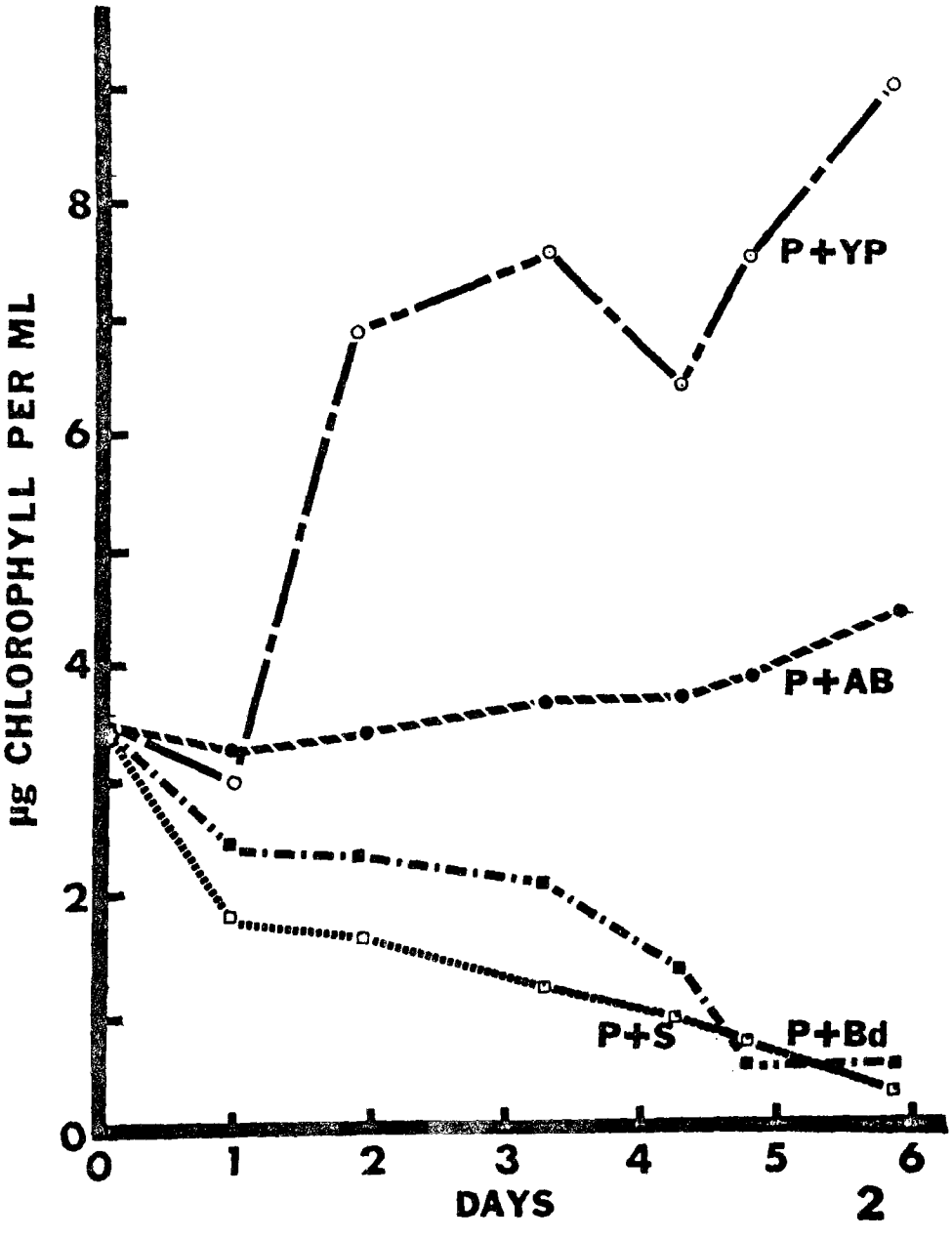
µg CHLOROPHYLL PER ML
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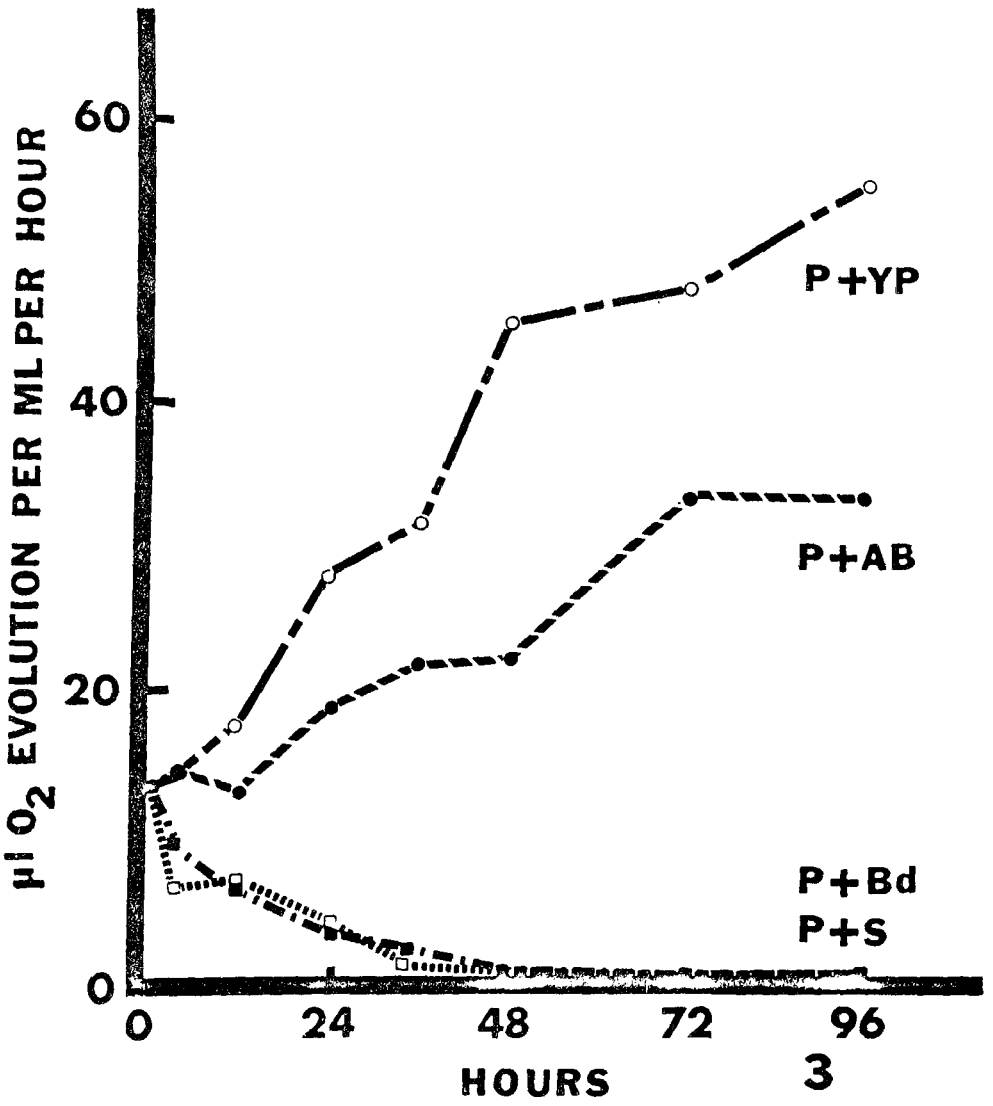
μl O2 EVOLUTION PER ML PER HOUR
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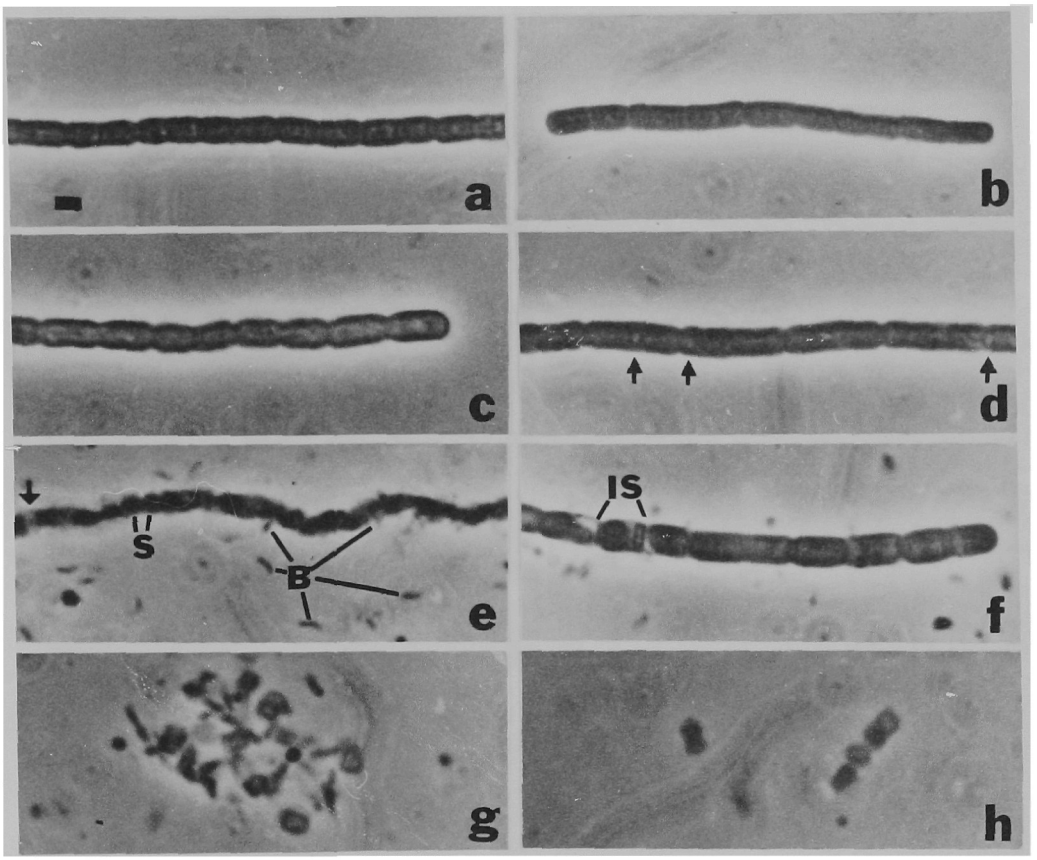
3



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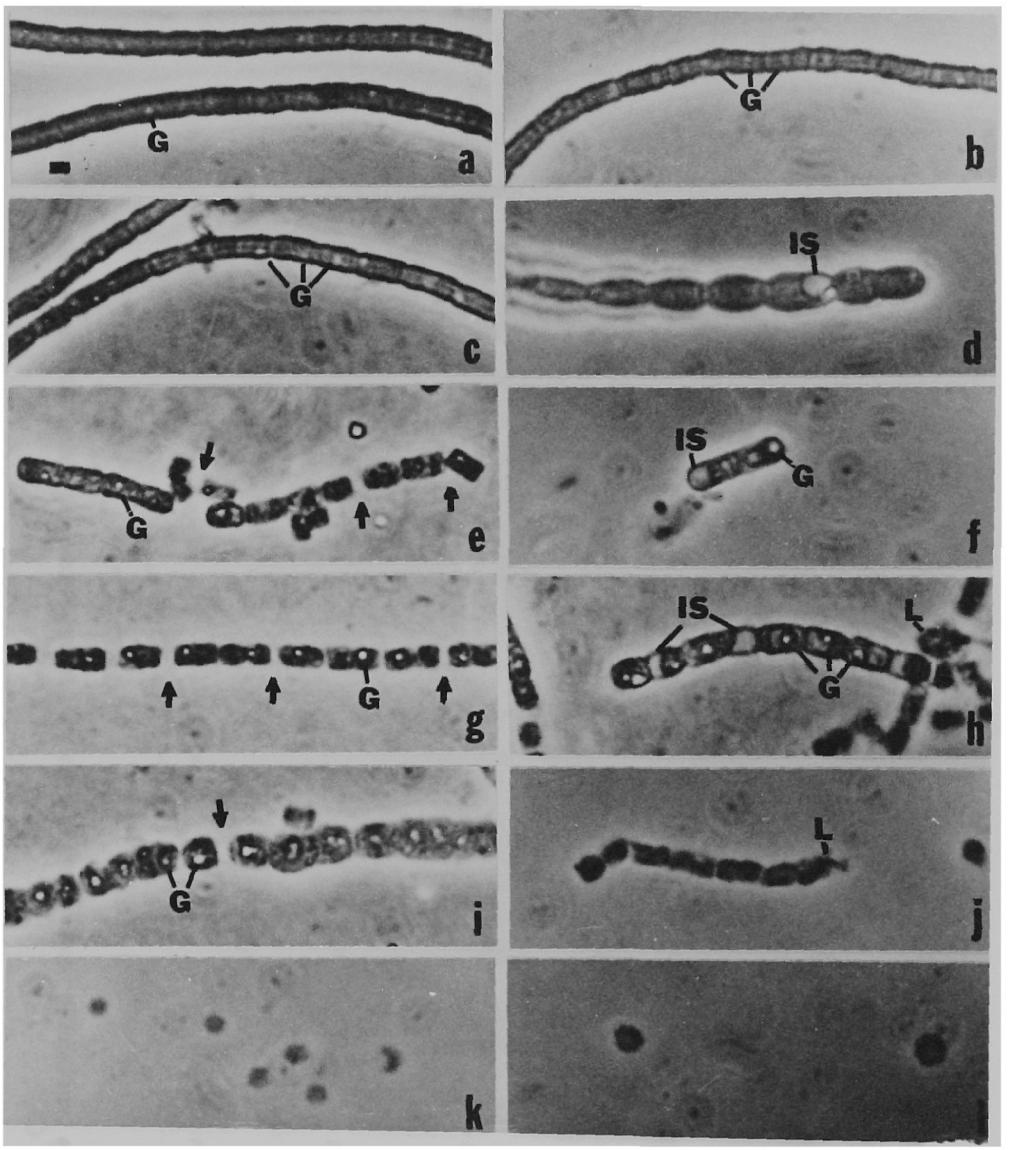

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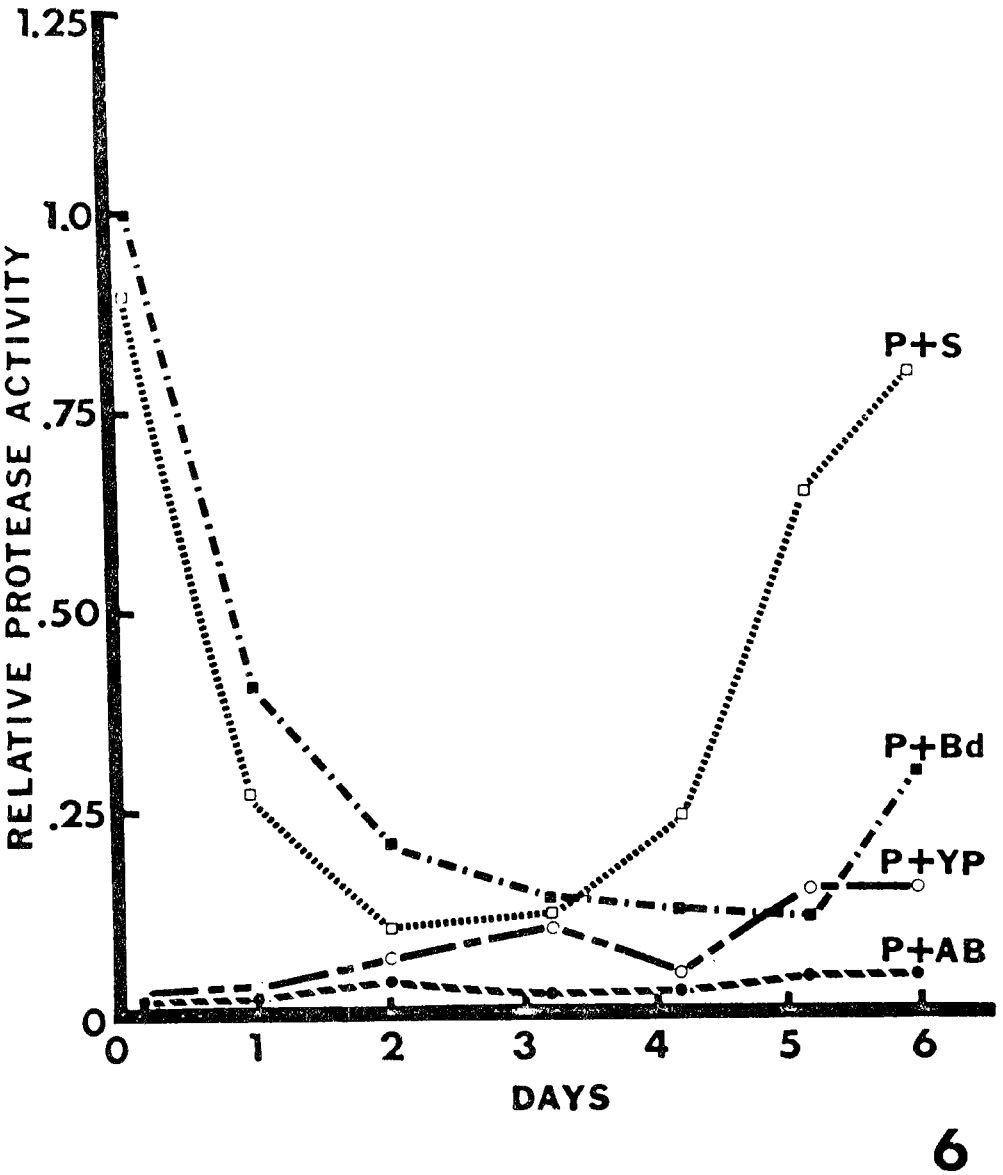
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RELATIVE PROTEASE ACTIVITY
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P+Bd
P+YP
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6

APPENDIX II

AN IMPROVED METHOD OF CELL ENUMERATION FOR FILAMENTOUS ALGAE AND BACTERIA

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Thomas Stetak
James Boulger

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May, 1973

A NOTE

(Revised Manuscript)

AN IMPROVED METHOD OF CELL ENUMERATION FOR FILAMENTOUS ALGAE AND BACTERIA

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ABSTRACT

A new simple method for estimating the number of individual cells per ml in suspensions of filamentous microorganisms is described. This two part procedure utilizes a standard microscopic counting chamber and is independent of filament length or individual cell size. A statistical analysis of the method is also presented.

It is extremely difficult to accurately count those microorganisms which are organized as multicellular filaments rather than as discrete single cells. This problem is magnified in studying the filamentous blue-green algae because they often grow to extreme lengths. The normal counting procedures which utilize either the Petroff-Hausser Counting Chamber or other hemocytometers (4,7), are usually inaccurate as well as tedious because the filamentous microorganisms are not randomly oriented but instead are linked in variable numbers. Although filamentous cells have been quantitated by a variety of methods (5,6,8,9), a direct and simple microscopic counting procedure has not been available. The method that follows simplifies studies of filamentous algae and bacteria.

The blue-green algal species, Phormidium luridum var. olivacea was the model microorganism utilized in this study, but any filamentous microorganism with visible cross walls separating individual cells can be employed. The technique, for example, would allow easy quantitation of cell numbers for common filamentous bacteria such as Streptococci or Vitreocilla. The algae were grown on Bacto Algae Broth (Difco) in 300 fc light intensity at 24° C. For counting purposes, samples were taken directly from this culture without washing or other treatment.

PREPARATION OF SAMPLE

The culture to be counted is shaken thoroughly in order to suspend settled trichomes or filaments. One small drop of the culture is placed in the center of the 400 square grid of a Petroff-Hausser Bacterial Counting Chamber (C.A. Hausser and Son) (Fig. 1). A flat coverslip supplied with the chamber is then placed over the sample, and tapped lightly. The suspension is allowed to settle for approximately one minute. (Note: If the culture is placed at the edge of the chamber, erratic results can occur due to the inability of long filaments to spread evenly from the irrigation groove.)

FILAMENT COUNTING PROCEDURE

Step I. All cells (from any filament or part of a filament) are counted within each of ten squares selected at random from the total of 400 squares in the chamber, and the total number of cells counted is designated as (C). Fig. 2 illustrates part of one such square and shows that the individual cells of Phormidium trichomes can be counted easily under proper optical illumination.

Step II. The 400 squares in the chamber are organized into 25 blocks of 16 squares per block. Fifteen blocks (normally the top, middle and lower rows of five blocks) containing a total of 240 squares are examined. The total number of squares that contain any part of a filament are termed (B). Step II is illustrated in Fig. 1. For example, in the block offset by arrows, 5 of 16 squares contain filaments or parts of filaments. (Note: When the phase contrast condenser of an A O series 10 microscope is left in place following Step I and used in conjunction with a standard brightfield 10X objective the resultant darkfield illumination greatly facilitates filament recognition.)

CALCULATIONS

The fraction of squares that contain filaments is obtained by dividing the term (B) by 240 (the total number of squares in 15 blocks). When $\frac{B}{240}$ is multiplied by the term ($\frac{C}{10}$), the mean number of cells in filament-containing squares, the mean number cells per chamber square $\frac{CB}{2400}$ is obtained. Each counting chamber has a Volume Constant which enables conversion from cells per square to cells per ml (for the Petroff-Hausser Chamber this is 3×10^9). Therefore, $\frac{CB}{2400}$ x Volume Constant equals cells/ml.

For each population that is counted, the above procedure should be carried out two or more times to statistically minimize the errors of sampling and of specimen dispersion.

There are many difficulties in statistically evaluating this technique. Although counting chambers have been used to quantitate filamentous microorganisms (1,5,6,9), the methodology of these studies is obscure. As a practical solution, we have compared our method with two other procedures by the following techniques:

1) To check the accuracy of our new method, direct counts were made of every cell in all 400 squares of the counting chamber. Multiplication of this total by the chamber volume constant was the most accurate means of determining the numbers of cells in a sample, but was enormously time consuming and tedious.

2) Another accurate, but time consuming technique utilizes measurement of both the length and number of filaments in the chamber (9). The average filament length was divided by the average length of a single cell and this result was multiplied by both number of filaments in the chamber and the volume constant. As stated by Padan and Shilo (8) this method is very difficult to carry out when long filaments are present.

Other methods employed standard curves to correlate number of cells per ml to, a) dry wt of the sample, and b) chlorophyll a concentration per sample (10). Dry wt analyses which have been used by Halfen and Francis (3) are very time consuming and relate to cell volume as much as to cell number. Thus, in our system dry wt is neither an accurate nor an effective method.

Quantitative estimates of cell number based on optical densities are only accurate for carefully defined "normal" growth conditions because temperature, age, light intensity and other environmental factors such as selenium concentration directly affect pigmentation, (1,3, unpublished data).

Each of the counting chamber techniques described above was statistically compared with our new method. Optical density was used as a comparative standard only under the "normal" growth conditions described for routine growth of P. luridum.

A. As anticipated, the rank-order correlation (2) between our procedure and the "filament length" technique was very high (rho=0.7441, t=4.4552, 16df, $p < .001$) for a sample of eighteen counts. For this same sample, the correlation between the "filament length" technique and optical density readings was 0.7147 (t=4.0873, 16df, $p < .001$) while the correlation between the new method and optical density was 0.9592 (t=13,5706, 16df, $p < .0001$). For a larger sample (N=54), the rank-order correlation between the estimate by the proposed method and optical density reading was 0.9432 (t=20.4726, 52df, $p < .0001$). This is graphically presented in Fig. 3 for N=36. It is clear that under normal cultural conditions both methods are very highly correlated with optical density readings but that the new method of cell enumeration is somewhat more accurate when optical density is used as a standard.

B. The new method was compared to the "filament length" method for twenty-five samples from one optical density level (OD=0.48). There was no statistically significant difference (2) between the means of the two methods (t= -1.2118, 23df, $p < .10$). The standard error of measurement was slightly larger for the "filament length" method when compared to the new method (SE's were 1.0410 and 0.8677 respectively).

C. Nine samples were selected from each of three optical density levels. A comparison was made of the mean difference between the total number of cells as predicted by the new counting method and the actual number of cells determined using comparison method #1 described above. This analysis revealed no statistically significant difference between the estimate by the proposed method and the true number of cells (t=0.0557, 8df, $p < .10$).

On the basis of these comparisons, it is clear that the "filament length" and the proposed method are quite comparable in their estimation capabilities.

Additionally, it is clear that the proposed method is accurate when estimates are compared with actual cell counts. The relative ease of the new technique and the accuracy of its predicted values indicate that it will be quite useful in further investigations and in routine cell enumeration with filamentous algae and bacteria.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Owens-Illinois Corporation, Toledo, Ohio; an Ohio Water Resources Allotment Grant (A-025-Ohio); and a General Research Support Grant from the National Institutes of Health (#5-S01-RR05700-02). The suggestions and improvements of this manuscript by R. Watterson and E. H. Freimer are appreciated.

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LEGEND

Fig. 1

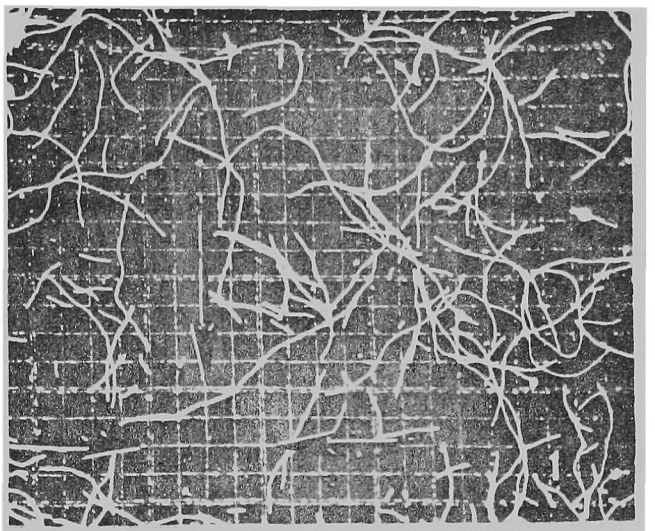
Low power darkfield micrograph of a Phormidium culture placed on the four hundred square grid of a Petroff-Hausser Counting Chamber illustrating the viable length filaments that are easily quantitated with this new procedure. Arrows point to one of the blocks of 16 squares described in the text. Magnification 110X.

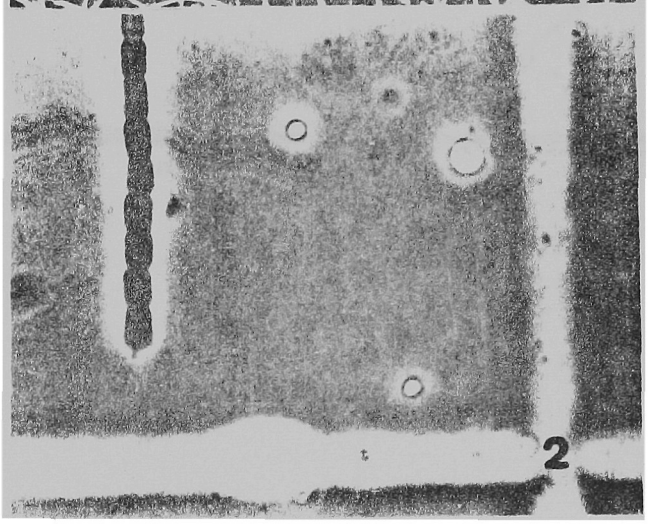
Fig. 2

Phase contrast micrograph part of one of the four hundred squares showing the individual cells comprising the Phormidium trichome. All of the individual cells in any ten filament-containing squares must be counted in the first part of the counting procedure. Magnification 3300X.

Fig. 3

Phormidium cell concentration in relation to culture absorbance at 610 nm demonstrating the wide absorbance range over which the counting procedure is applicable. Method of enumerating cells is described in the text.

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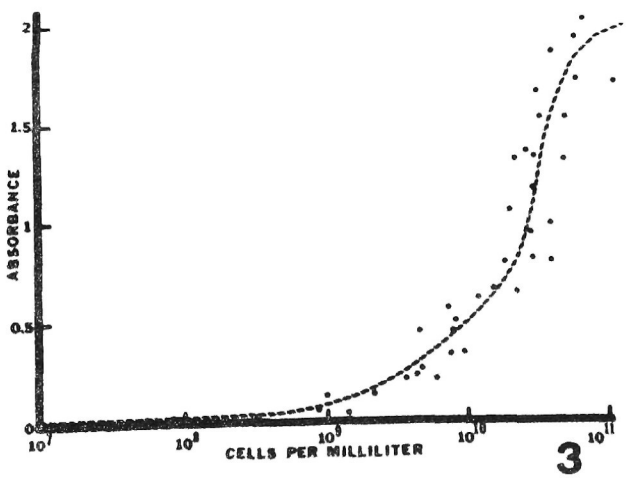
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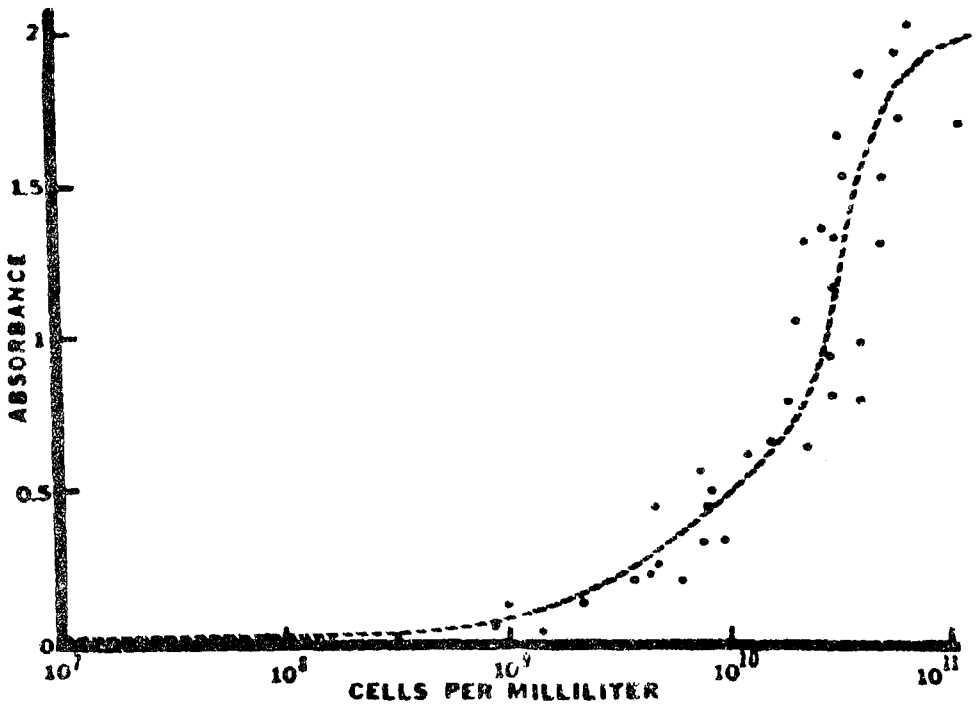
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APPENDIX III

THE EFFECT OF SELENITE ON THE PHYSIOLOGICAL AND MORPHOLOGICAL PROPERTIES OF THE BLUE-GREEN ALGA, PHORMIDIUM LURIDUM VAR. OLIVACEA

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June, 1973

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The Effect of Selenite on the Physiological and Morphological Properties of the Blue-Green Alga, *Phormidium luridum var. olivacea*

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Toledo, Ohio 43614

ABSTRACT

Phormidium luridum cultures were treated with sodium selenite in concentrations ranging from 10^{-6}M to 10^{-2}M. Physiological and morphological observations revealed the toxicity of selenite to be greater at the higher concentrations. Photosynthetic activity was inhibited within 6 hr in all cultures except control and 10^{-6}M selenite. In contrast to the increasing culture turbidity of control and 10^{-6}M selenite cultures, the turbidity of the other experimental selenite cultures declined in proportion to time and selenite concentration. Chlorophyll extraction revealed similar results. Phormidium cultures treated with selenite concentrations greater than 10^{-6}M showed a gradual loss of the bright green color and turned semi-transparent. Granules of reduced selenium were observed in the flasks of higher selenite concentrations. Light microscopy revealed these granules to be associated with the cells. Other structural changes observed in conjunction with the physiological changes included the presence of intracellular and intercellular spaces, spheroplast formation, and gradual cell lysis. Protein analyses of total cell samples and supernatant fractions confirmed cellular breakdown of selenite treated algal cells.

INTRODUCTION

Microorganisms have been found to respond to selenium in a variety of ways. Growth inhibition by selenium compounds is well known as has been reported in bacteria (11, 15), yeasts (4), and algae (12, 5). Conversely, a few organisms have exhibited a tolerance to selenium compounds of various concentrations as have been demonstrated in Salmonella (1), Escherichia coli (16), Anacystis nidulans (6), and Chlorella vulgaris (14).

In general, however, selenium and its derivative compounds are considered quite toxic and actually have become objects of increasing environmental concern (3).

Because we are interested in determining the effects of a variety of pollutant toxic elements, such as selenium, on the structure and physiology of the blue-green algae, we have examined the interaction of selenite with the blue-green algal species Phormidium luridum.

The response of the photosynthetic system to selenite exposure has not yet been elucidated. We believe that because of the photoautotrophic nature of these algae, photosynthesis would be both the most interesting and most significant physiologic process to examine in relation to the cells' reaction to selenite. If selenite involvment is coupled directly to photosynthetic ability then the analysis of the cells' response can be much more rapid than the density measurements of cell growth previously employed (5, 6).

A recent comparative survey of the toxicity of four selenium compounds to two species of Blue-green algae (5) demonstrated that the mechanisms responsible for the toxic effects differed in the two species of algae and that the degree of growth inhibition varied with the form of selenium tested. Selenite was found to be more inhibitory to growth than selenate or the organic selenium compounds. Of the two species studied, Anacystis nidulans appeared to be three times more

tolerant to selenite than Anabaena variabilis.

Changes in morphology of cells treated with selenium compounds have been reported in Chlorella vulgaris (12), Candida albicans (9), Penicillium notatum (10). Exposure to selenomethionine was found to inhibit the division mechanism in C. vulgaris, producing "giant" cells. After a short adaptation period, however, the cells resumed normal growth. An opposite effect of stimulated cell division was observed in C. albicans. In addition, the filamentous form of this fungus was found to revert to a yeast-like budding form in the presence of 10^{-4}M and 10^{-5}M selenite. A recent investigation by Pitt (10) revealed striking morphological changes in P. notatum when treated with selenite concentrations of 10^{-3}M and 10^{-4}M. Enlarged terminal and intercalary hyphal segments, grossly deformed hyphae and sporophores were observed.

Specifically the present report relates the toxic effects of selenite on some physiological properties, particularly photosynthesis, of the blue-green alga, P. luridum, and the concomitant morphological changes observed in the cells.

MATERIALS AND METHODS

Experimental Organism: Phormidium luridum var. olivacea (Species #426) was obtained bacterial-free from the Culture Collection of Algae at Indiana University.

Media and Cultural Conditions: Stock cultures were grown in 500 ml Erlenmeyer flasks containing a 10% inoculum and 100-150 ml Algae Broth (AB) (Difco). Incubation with continuous rotary agitation was at 28 C under constant 300 fc illumination. Transfers to fresh media were made weekly.

Large yields of P. luridum were obtained by culturing in a Microferm Fermentor (New Brunswick) at a constant temperature (30C), controlled light intensity, continuous aeration (2 l air/min), and agitation (200 rpm of the paddle blades). Cells were harvested after three to seven days, depending on the amount of original inoculum and desired culture density.

Stock solutions of 2×10^{-2}M sodium selenite (Alfa Inorganics) were prepared aseptically by filtering through a 0.45 um Millipore Filter (Millipore Corp., Bedford, Mass.). Serial dilutions, under sterile conditions, were made from the 2×10^{-2}M sodium selenite stock to botain stock solutions of 2×10^{-3}M, 2×10^{-4}M, 2×10^{-5}M, and 2×10^{-6}M sodium selenite. The experimental growth medium was obtained by mixing equal volumes of the selenite stock solutions with double strength AB, thereby, reducing the molarities of the selenite media by one half.

Assay Procedures: Growth was assayed spectrophotometrically in side arm flasks (Bellco) at 610 nm on a Coleman Junior II Spectrophotometer.

A modified chlorophyll extraction method of Cohen-Bazire (2) was employed, i.e., one hour extraction in 100% methanol. Absorbance was recorded at 660 nm on a Gilford Model 2400 Recording Spectrophotometer.

Photosynthetic activity was measured as oxygen evolution with a Gilson Differential Respirometer. Samples of cell suspensions (3.0 ml) were removed from the culture flasks, centrifuged and resuspended in carbonate-bicarbonate buffer (Mixture #9) (15). Oxygen evolution (ul) was recorded at fifteen minute intervals for one hr.

Dry weights of cells were determined after drying washed 10 ml samples in a 120 C oven for 72 hr. Net weights were expressed in mg/ml.

Protein levels were determined by the Lowry procedure (7) for both total cell samples and supernatant fractions. Transmittance was recorded on a Klett-Summerson Colorimeter at 660 nm.

Light Microscopy: Cultures were observed periodically by phase-contrast microscopy. A drop of the unfixed cell suspension was placed on a slide and the edges of the coverslip sealed with Vaspar, a mixture of paraffin and vaseline. A Zeiss Universal Photomicroscope, equipped with a Nikon automatic exposure meter and camera attachment, was used to photograph the cells. All micrographs were taken with the Zeiss microscope strobe flash attachment on either Kodak Tri-X or High Speed Ektachrome film.

RESULTS

Effect of Selenite on the Growth of P. luridum: A comparison of the turbidity of P. luridum cultures in varying concentrations of selenite media is illustrated in Figure 1. Control and 10^{-6}M selenite culture turbidity increased in parallel almost five-fold over a period of 184 hr. The turbidity of 10^{-5}M selenite flasks increased gradually for 65 hr and thereafter declined slowly. Turbidity increased slightly during the first 24 hr in both 10^{-4}M and 10^{-3}M selenite cultures, followed by a stationary period up to 96 hr, and thereafter decreased steadily. Cultures in 10^{-2}M selenite media showed a three-fold loss of turbidity for the duration of the experiment.

In order to determine that spectrophotometric measurement of growth was not due solely to a change in pigment, dry wt of cells were obtained in conjunction with the turbidity measurements. Figure 2 illustrates the change in dry cell wt for the experimental period. A linearly increasing trend is observed for control and 10^{-6}M selenite cultures, similar to the growth curves. On the contrary, P. luridum cultures treated with higher selenite concentrations declined in dry cell wt proportionately with time and concentration.

A significant change in pigment was observed accompanying the loss of culture turbidity: controls and 10^{-6}M selenite cultures turned a darker green and became more dense with time; the 10^{-2}M, 10^{-3}M, 10^{-4}M, and 10^{-5}M selenite cultures exhibited a loss of the bright green color, turning semi-transparent with time. In addition, at the termination of the experiment, dark red granules, assumed to be reduced selenium, were observed in cultures containing higher selenite concentrations.

Effect of Selenium on Chlorophyll: Chlorophyll extractions revealed a direct relationship between an increase or decrease in turbidity and pigment per

milliliter cell suspension. Figure 3 illustrates the change in total chlorophyll of control and experimental P. luridum cultures. A three-fold net loss of chlorophyll was exhibited by the 10^{-5}M selenite cultures, whereas the 10^{-4}M, 10^{-3}M, and 10^{-2}M selenite cultures declined in chlorophyll content proportionally to the loss of culture turbidity.

Effect of Selenite on Photosynthesis: Control and 10^{-6}M selenite cultures revealed a steady increase in evolved oxygen over the experimental period (Figure 4). At six hr, the volume of oxygen produced by the selenite-treated cultures appeared to decrease in proportion to increasing selenite concentrations. Photosynthetic activity of all selenite cultures, with the exception of 10^{-6}M, ceased at 24 hr. Concomitantly, growth as indicated by measurement of culture turbidity (Figure 1) and dry wt/ml (Figure 2) essentially ceased in comparison to control cultures. The slight initial decline in control and 10^{-6}M selenite flasks represents the dilution of the cultures at the time of inoculation. All curves stem from a single point on the graph which represents oxygen evolution of the original inoculum.

Effect of Selenite on Cell Protein Content: Figure 5 illustrates the change in protein concentration present in the supernatant fractions of the Phormidium cultures. No significant protein could be detected in any samples up to 20 hr. At 40 hr, however, a small amount was measured in all experimental and control flasks. Thereafter, as shown from the graph, the protein concentration was consistently greater in the supernatant of the higher selenite concentrations. The protein concentration increased in the supernatant fluid and correspondingly decreased in the remaining cellular fraction.

Light Microscopy: Phase-contrast microscopy revealed distinct morphological

changes in selenite treated cells. Figure 6a shows a filament from a 48 hr old control AB culture. Individual cells within the trichome are generally uniform in size, having a width of about 2 um. P. luridum cells cultured in 10^{-6}M selenite media (Figure 6b) revealed no morphological alterations and resembled control cells.

Examination of cells treated with 10^{-5}M selenite for periods up to 48 hr revealed no noticeable structural changes. Figure 6c illustrates a typical filament of a 60 hr 10^{-5}M selenite culture. The trichomes were found intact but cells appeared swollen and began to show intracellular spaces. By 96 hr (Figure 6d) intracellular vacuoles were commonly observed and in some cases occupied the entire cell space. Cell lysis was observed at 120 hr and the formation of protoplasts was also frequently seen (Figure 6e).

In the 10^{-4}M selenite cultures, structural changes were noted within 48 hr. Fragmentation of the filaments into small but still intact chains was the first apparent sign of breakdown. Individual cells were more distorted, many appeared barrel-shaped or rounded up and frequently small yellow refractile granules were observed within the cells (Figure 6f). These granules are thought to be aggregations of photosynthetic pigments which migrate and accumulate in the central area of the cell. Protoplast formation and lysis was observed by 112 hr (Figure 6g). Occasionally skeleton trichomes as seen in Figure 6h were found among the cell debris.

A more rapid progression of cell breakdown was observed in cultures treated with 10^{-3}M and 10^{-2}M sodium selenite. Microscopic examination of these cells revealed the presence of red selenium granules associated with the cells (see arrows Figure 6i). These granules appeared to be located intracellularly as well as attached to the outer surface of the filaments. The presence of such granules has been reported previously in blue-green algae (5) and Penicillium notatum (10). It is thought that these granules are of a similar

nature to the red deposits observed in the culture medium. Intercellular spaces preceding fragmentation were again seen in filaments by 48 hr. By 72 hr individual cells had rounded up into protoplast forms with vacuolar areas appearing to occupy a large part of many of the protoplasts. The rounded-up cell forms were seen breaking out of the intact filament (Figure 6j) before their eventual lysis.

DISCUSSION

Toxicity of selenium to blue-green algae has been reported previously (5) suggesting different modes of action for the two species studied. This report, however, took into consideration the interaction of the sulfide ions to evaluate the extent of damage caused by the selenium compounds. The results of our investigation clearly confirm the toxic nature of selenite even in the presence of 4.26 mM sulfate in the growth medium.

Photosynthesis was found to be inhibited almost immediately after exposure to selenite. This data confirms that oxygen evolution is far more rapid and resistive parameter for measuring the response of blue-green algae to selenium compound. Inhibition of photosynthesis was accompanied, following a short lag period, by a gradual degradation of the photosynthetic pigments. This was observed visually in the culture flasks by a progressive bleaching from the deep green color to a semi-transparent yellow. The spectrophotometric analyses of extracted chlorophyll support the pigment degradation.

It is possible that selenite exerts its toxic effect on photosynthesis so rapidly by interfering with an enzymatic system with the selenite ion becoming reduced in the process. The presence of the selenium granules in the culture medium gives support to this hypothesis. Other investigators have reported similar red granules which have been identified as elemental selenium (5, 10), A report by McCready, Campbell, and Payne (8) explaining growth inhibition has suggested that selenite becomes reduced to elemental selenium when it oxidizes sulfhydral enzymes. Shrift (13) also has suggested that the oxidizing properties of the selenite ion may be responsible for inhibitory damage. It would appear that a similar mechanism might be occurring in P. luridum to account for both the granules of reduced selenium and inhibitory effect on the cells' photosynthesis.

Light microscopic observations revealed that loss of culture turbidity, photosynthetic activity, and chlorophyll was accompanied by gradual cellular breakdown which terminated in lysis. During cellular breakdown, filaments were observed to fragment and individual cells rounded up into spheroplast forms. The time required for cell lysis was dependent on the concentration of selenite.

Unfortunately the large cells (14) we were hoping would form due to the cells response to selenite never materialized. Analysis of the photosynthetic data would certainly give evidence that this was not possible as it appeared that photosynthesis was the most sensitive physiological system to selenite concentration. Without energy and carbon assimilation cellular development to the level necessary to form giant cells would not be possible. Electron microscopic examination of these cells is underway to determine if subcellular pathology, particularly in the photosynthetic system, is evident.

Whether blue-green algae are capable of accumulating and retaining sub-lethal doses of selenite (between 10^{-6}M and 10^{-5}M) is not presently known. Copeland (3) has found that zooplankton in Lake Michigan is capable of assimilating and retaining up to twelve times the amount of selenium found in the lake sediment. Blue-green algae are common in aquatic habitats and play a vital role in the food chain. The potential danger of P. luridum greatly concentrating a toxic element in the food chain is reduced by the apparent sensitivity of this Blue-green alga's physiological and structural integrity to selenite exposure.

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LEGEND

Fig. 1. Effect of sodium selenite concentrations on the growth of P. luridum. Absorbance measured at 610 nm. Conditions as described in Materials and Methods. Symbols: (⊙——⊙) control culture grown in AB medium; (□·····□) AB + 10^{-6}M selenite; (○·–·○) AB + 10^{-5}M selenite; (△– – –△) AB + 10^{-4}M selenite; (▣–▪–▣) AB + 10^{-3}M selenite; (△–▬–△) AB + 10^{-2}M selenite.

Fig. 2. Effect of sodium selenite concentrations on the increase in cell mass (dry wt) of P. luridum cultures. Growth conditions as described in Materials and Methods. Symbols as in Fig. 1.

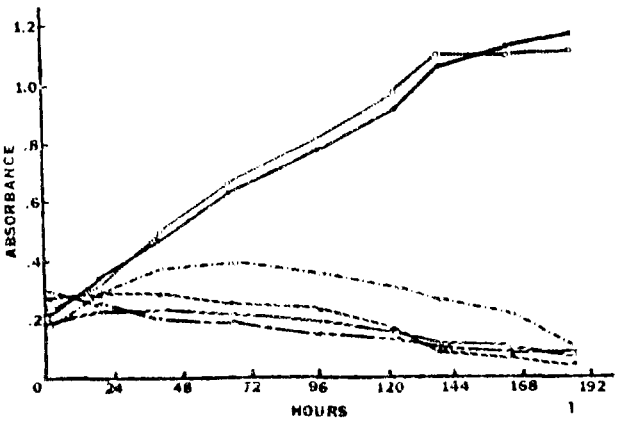
Fig. 3. Comparison of chlorophyll content of P. luridum cultures treated with sodium selenite. Extracted chlorophyll measured at 660 nm. Symbols as in Fig. 1.

Fig. 4 Photosynthetic activity (evolved oxygen) of P. luridum cells treated with sodium selenite. Culture designations as in Fig. 1. Essentially all photosynthetic capability of the algae exposed to 10^{-5}M or more selenite was lost in less than 24 hr.

Fig. 5. Protein concentration in P. luridum culture supernatant fluid following treatement with sodium selenite. Culture designations as in Fig. 1.

Fig. 6. Phase contrast photomicrographs of P. luridum showing the effect of varying concentrations and treatment times of sodium selenite on cell structure. (a) 48 hr control cells grown in AB medium; (b) no effect noticeable after 120 hr in AB + 10^{-6}M selenite; (c) 60 hr in AB + 10^{-5}M selenite caused some swelling and development of intracellular spaces; (d) 96 hr in 10^{-5}M selenite was characterized by the development of

large intracellular vacuoles; (e) lysis and protoplast forms were common after 120 hr in 10^{-5}M selenite; (f) yellow refractile granules in swollen cells were common after 48 hr in 10^{-4}M selenite; (g) protoplast formation within the still rigid cell wall and cell lysis were common at 112 hr; (h) after 112 hr complete skeleton trichomes, as shown here, could be seen in 10^{-4}M selenite; (i) in the higher concentrations of selenite (10^{-3}M, 10^{-2}M) red granules of reduced selenium could be seen after 24-48 hr in close association with the cell, usually near the cell septa. Their intracellular nature was not ascertained; (j) 72 hr in 10^{-3}M selenite resulted in cells rounding up, breaking out of the trichome and lysing.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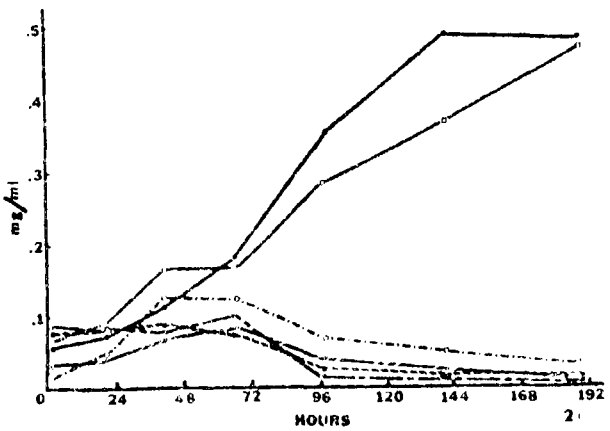
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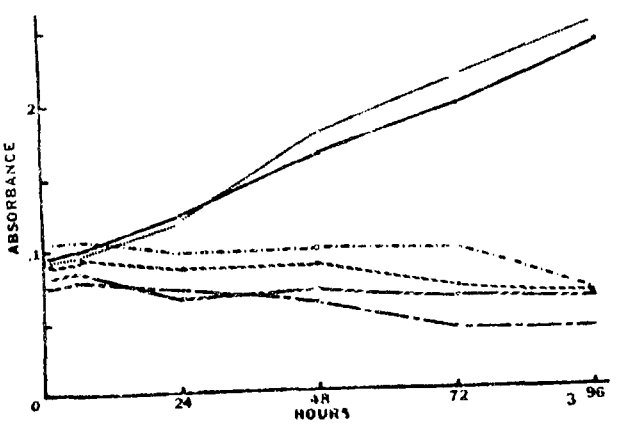
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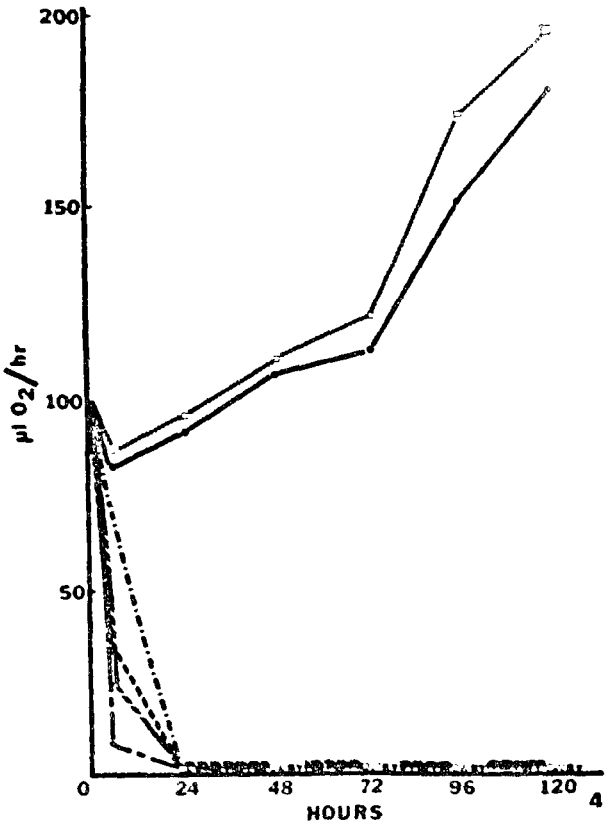
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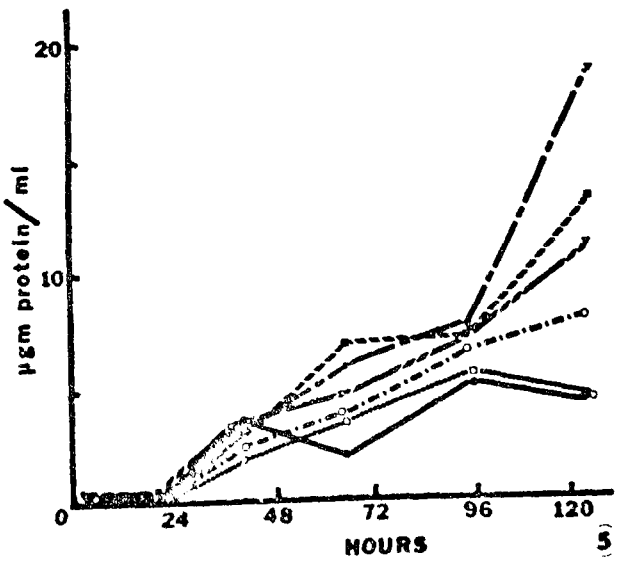
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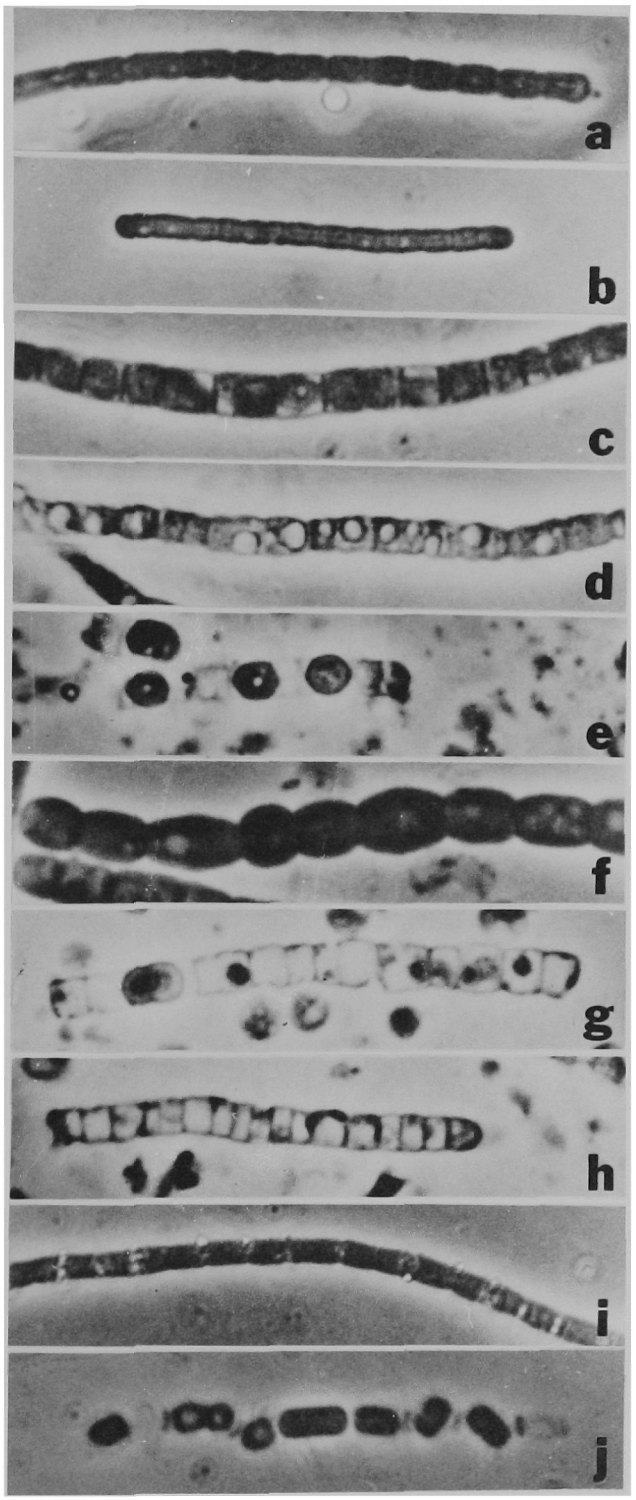
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